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August 1979

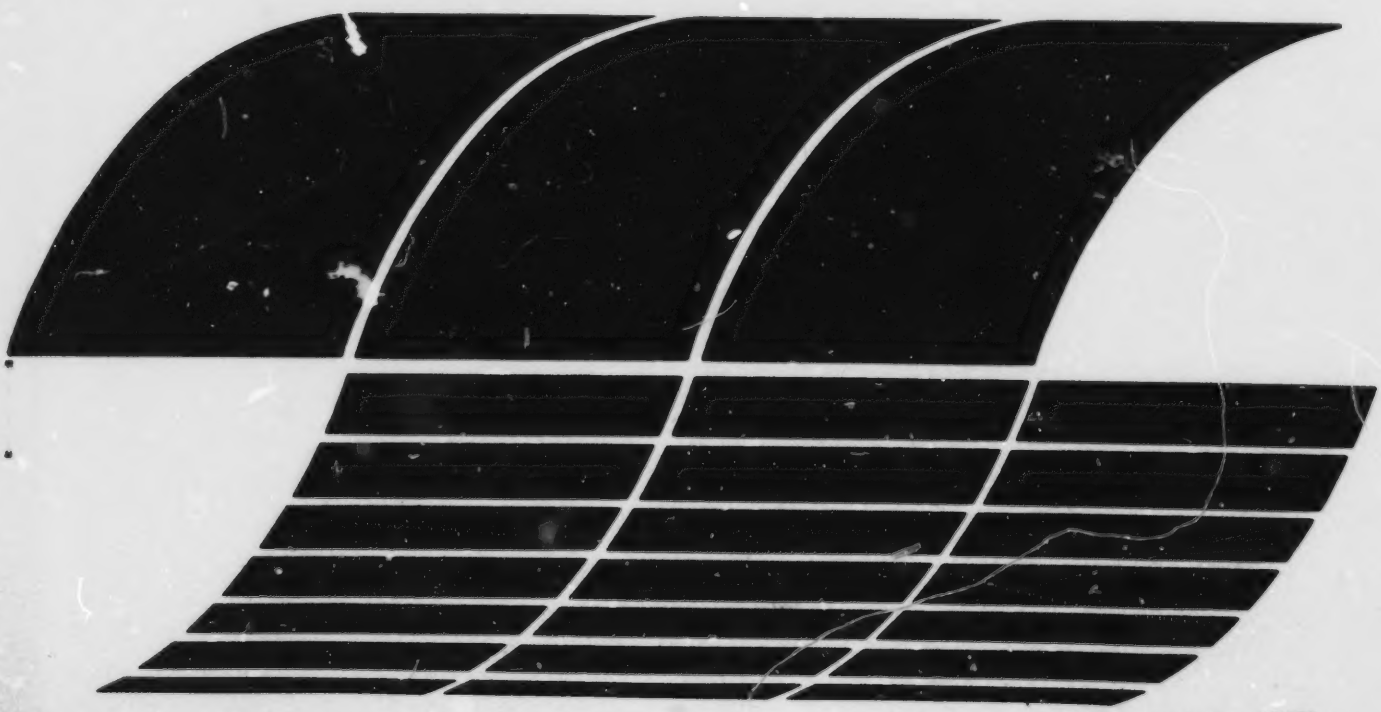
Research and Development

# Mutagenicity Testing in Mammalian Cells

## Multiple Drug-Resistance Markers

MASTER

## Interagency Energy/Environment R&D Program Report



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# MUTAGENICITY TESTING IN MAMMALIAN CELLS:

## Multiple Drug-Resistance Markers

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## FOREWORD

Short-term toxicologic testing, particularly methods for detecting genetically active substances, has become a keystone of the Environmental Protection Agency's efforts to detect and evaluate environmental hazards. Among the most serious hazards to man's future are agents that damage the genome, or the information bank of cells and organisms, by mechanisms that can increase the future population load of inherited structural defects and genetic diseases or that can increase the incidence of cancer in the current generation. Tests for environmental mutagens play a dual role by virtue of their ability to detect mutagens and their presumptive ability to detect carcinogens, owing to abundant laboratory evidence that most known carcinogens respond positively to tests for mutagens.

The Biomedical Sciences Division of the Lawrence Livermore Laboratory has for the past five years been heavily engaged in the development and application of short-term tests for genetic toxicology. Specific examples are as follows:

- basic studies of classes of mutations in mammalian cells in culture and methods for selective recovery of mutant cells;
- development of suitable strains of cells for detection of forward mutations at multiple loci, and of detailed experimental protocols for mutagen detection;
- development and application of multiple modes of testing *in vitro* and *in vivo* for chromosomal damage and misrepair in the form of sister chromatid exchange, together with simultaneous correlation with mutation induction;
- development and application of highly sensitive tests for injury to sperm cells in the male testis and to oocytes in the juvenile female ovary;
- development of automated cytochemical methods for detection of rare events of mutation and malignant transformation of cells in living animals, including man;
- application of cytogenetic and automated cytopathologic methods to workforce populations engaged with energy technology, and

- in collaboration with our Environmental Sciences Division, application of a battery of the foregoing short-term tests to complex effluents from coal gasification and oil shale retorting technologies.

The following report describes the development and partial validation of one of the major tests in our genetic toxicology test battery. A strain of cultured Chinese hamster ovary (CHO) cells has been selected that is capable of recognizing mutations in four independent genes. All parameters that must be controlled for reproducible quantitative assays have been validated with a set of direct-acting mutagens. Work now in progress will extend the assay to chemicals requiring metabolic activation and to partially fractionated complex effluents. This assay is a leading contender among reliable mammalian cell mutagenesis tests that should be applied when appropriate to confirm the bacterial screening tests, and to verify that the genetic damage suggested by a screening test also occurs in the cells of higher organisms.

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## DISCLAIMER

This report has been reviewed by the Lawrence Livermore Laboratory, University of California, and the U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

## PREFACE

Short-term mutagenesis assays are expected to play a major role in identifying potentially toxic or mutagenic agents in the environment. A tier concept has evolved that relates the extent and rigor of the screening protocols to the expected level or extent of human exposure. While the first tier uses lower organisms for rapid, cost-effective prescreening, mammalian cells are desirable at the second level of testing because microbial and mammalian cells differ in their uptake and metabolic modification of many mutagens, and there are marked differences in the structure, organization and repair of procaryotic and eucaryotic genomes. Moreover, mammalian assay systems evaluate different portions of the genome and assay different types of genetic damage. Assays for forward mutation at several loci might be expected to respond to most types of genetic damage, minimizing false positive and negative tests. The latter point is of major importance; while many pure compounds have been tested, the frequency of false positives and negatives is virtually unknown for complex organic mixtures or crude effluent samples.

As an in vitro test system, Chinese hamster ovary (CHO) cells combine the favorable growth characteristics, e.g., high plating efficiency and rapid growth in either monolayer or suspension culture, necessary for large-scale cost-effective mutagen screening, with a near-diploid genome that facilitates genetic analysis. Direct drug-resistance selection procedures, such as those for mutants with alterations in genes coding for various salvage enzymes in purine or pyrimidine metabolism, allow quantitative mutagenicity testing at specific well-defined genetic loci. We have developed a CHO cell line that can be monitored for forward mutations simultaneously at four gene loci: the autosomal recessive aprt (adenine phosphoribosyltransferase) and tk (thymidine kinase) genes, the widely used X-linked hgprt (hypoxanthine-guanine phosphoribosyltransferase) gene, and the co-dominant gene for Na-K-ATPase. This multiple-marker assay can now be used to assess two important areas: (1) determination of the quantitative dose-response relationships for toxicity and mutagenicity observed with compounds requiring metabolic activation and known to be present in environmental pollutant mixtures, and (2) development of methods to allow activation and assay of crude mixtures and effluents after only limited fractionation or purification.

## ABSTRACT

As a first step in the development of a multiple-marker, mammalian-cell mutagenesis assay system, we have isolated a Chinese hamster ovary (CHO) cell line that is heterozygous for both the adenine phosphoribosyltransferase (*aprt*) and thymidine kinase (*tk*) loci. Presumptive *aprt* $^{+/-}$ heterozygotes with intermediate levels of APRT activity were selected from unmutagenized CHO cell populations on the basis of resistance to low concentrations of the adenine analog, 8-azadenine. A functional *aprt* $^{+/-}$ heterozygote with $\sim$50% wild-type APRT activity was subsequently used to derive sublines that were also heterozygous for the *tk* locus. Biochemical and genetic characterization of one such subline, CHO-AT3-2, indicated that it was indeed heterozygous at both the *aprt* and *tk* loci. Chinese hamster ovary (CHO) cell lines heterozygous at both the adenine phosphoribosyltransferase (*aprt*) and thymidine kinase (*tk*) loci were used for single-step selection of spontaneous and induced mutants resistant to 8-azaadenine ($AA^r$), 6-thioguanine ($TG^r$), ouabain ($OUA^R$), or 5-fluorodeoxyuridine ($FUdR^r$). Mutation data are reported for direct mutagens (EMS, ethyl methanesulfonate; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; NQO, 4-nitroquinoline-1-oxide) and promutagens (DMN, dimethylnitrosamine; BP, benzo(a)pyrene) activated by rat liver homogenates. Significant dose-dependent increases in mutation frequency were observed for all four genetic markers after treatment of CHO-AT3-2 cells with ethyl methanesulfonate. Critical plating densities were established for $AA^r$, $TG^r$, and $FUdR^r$; no density dependence was observed for $OUA^R$. Expression of mutant phentotypes after mutation induction with EMS, DMN, or BP was optimal after 2 to 4 d for $AA^r$, 6 to 8 d for $TG^r$, 3 d for $OUA^R$, and 1 to 3 d for $FUdR^r$. The induced mutant frequencies as a function of relative cell survival after treatment with EMS, DMN, or BP showed locus-specific differences in sensitivity. Of 59 clonal isolates resistant to AA and assayed for APRT activity, 87% had $\leq$ 5% wild type activity; of 30 $TG^r$ clones assayed, 83% had $\leq$ 5% wild type HGPRT activity. Of 42 $FUdR^r$ clones assayed, 98% had $\leq$ 1% wild type TK activity. Fifty additional clones selected in medium containing FUdR displayed cross resistance to 5-bromodeoxyuridine (BUdR) and trifluorothymidine (TFT) and all were sensitive to HAT (hypoxanthine-amethopterin-thymidine) medium. The *tk* locus showed the largest mutational response as a function of cell survival after mutagen treatment. The rapid expression kinetics for $FUdR^r$ and the possibility that the locus detects a broader spectrum of genetic lesions than the other drug-resistance markers are discussed in terms of a sensitive screening assay for detecting potential mutagens.

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## List of Abbreviations

| | |
|---|---|
| α-MEM | alpha-minimum essential eagle medium |
| AA | 8-azaadenine |
| AG | 8-azaguanine |
| AMP | adenosine monophosphate |
| APRT | adenine phosphoribosyltransferase, EC 2.4.2.7 |
| ATP | adenosine triphosphate |
| BP | benzo(a)pyrene |
| BUdR | 5-bromodeoxyuridine |
| CdR | deoxycytidine |
| CHO | Chinese hamster ovary |
| DEAE | diethylamino ethyl cellulose |
| DFCS | dialyzed fetal calf serum |
| DMN | dimethylnitrosamine |
| EDTA | ethylenediamine tetraacetic acid |
| EMS | ethyl methanesulfonate |
| FCS | fetal calf serum |
| FUdR | 5-fluorodeoxyuridine |
| HAT | hypoxanthine-amethopterin-thymidine |
| HGPRT | hypoxanthine guanine phosphoribosyltransferase, EC 2.4.2.8 |
| IMP | inosine monophosphate |
| MNNG | N-methyl-N'nitro-N-nitrosoguanidine |
| 4-NQO | 4-nitroquinoline-1-oxide |
| $Na^+$-$K^+$ATPase | $Na^+$- and $K^+$-activated, $Mg^{++}$-dependent ATPase, EC 3.6.1.3 |
| OUA | ouabain octahydrate |
| PBS | phosphate-buffered saline |
| PE | mean plating efficiency |
| PEG | polyethylene glycol |
| PRPP | 5-phosphoribosyl-1-pyrophosphate |
| S | mean relative survival; surviving cell fraction |
| S.D. | standard deviation |
| SEM | standard error of the mean |
| TG | 6-thioguanine |
| TFT | trifluorothymidine |
| TK | thymidine kinase, EC 2.7.1.21 |
| WT | wild type cells |

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## SECTION 1

## INTRODUCTION

*In vitro* mutagenesis assays are expected to play a major role in identifying carcinogenic or mutagenic agents in the environment (8, 18,65). Because mechanisms for the generation and repair of mutagen-induced damage may vary considerably in procaryotic and eucaryotic systems, mammalian mutagenesis assays are needed to confirm and extend the microbial data. The use of multiple genetic markers may increase the reliability of such mutagenesis assays for those compounds having diverse types of interaction with genetic material.

The most thoroughly investigated mutant phenotypes of mammalian cells are drug-resistant mutants with presumptive alterations in genes coding for salvage enzymes in the purine and pyrimidine metabolic pathways (1,5, 10,13,14,17,22,24,25,26,35,38,45,44,49,52,58,69,70,74,75 78,80,86,92,96) Operationally, the definition of a drug resistant phenotype is a mutant's ability to survive in the presence of cytotoxic purine or pyrimidine analogs. Analogs frequently used include 6-thioguanine (TG) or 8-azaguanine (AG), which are metabolized by the salvage enzyme, hypoxanthine guanine phosphoribosyltransferase (HGPRT), or 8-azaadenine (AA) or 2,6-diaminopurine (DAP), which are metabolized by adenine phosphoribosyltransferase (APRT). Thymidine analogs such as 5-bromodeoxyuridine (BUdR), 5-fluorodeoxyuridine (FUdR), or trifluorothymidine (TFT) selectively kill cells with thymidine kinase (TK) activity. Ouabain (OUA), which specifically inhibits active transport by $Na^+$-$K^+$ ATPase, is also used to select drug-resistant mutants (6,19,27).

The presence of more than one functional allele for autosomal loci in mammalian cells makes direct, single-step selection of recessive, drug-resistance phenotypes impractical as a means of assaying induced mutations. Mammalian cell mutagenesis studies have, in most cases, used resistance to either 6-thioguanine (TG) or 8-azaguanine (AG) as a convenient, well-characterized, and selectable genetic marker to measure forward mutation at the X-linked hypoxanthine-guanine phosphoribosyl transferase (*hgprt*) locus (1,15,16,23,30,37,38,44-46,50,58,72-76,83,92,96). Systematic determination of optimal conditions for the phenotypic expression and selection of $TG^r$ mutants in Chinese hamster cells (15,16,37,48,72,73,74,75,83,92,96) as well as the development and application of a practical, *in vitro* CHO/HGPRT mutagenesis assay system by Hsie and co-workers (29,30,44-47,72-76), has facilitated quantitative analysis of mutation induction by a wide range of physical and chemical

agents. Comparable selection of APRT- or TK-deficient mutants requires the prior derivation of cells heterozygous at the autosomal recessive aprt and tk loci (17,25,26,52,80,86). Ouabain (OUA) resistance is a genetic marker that is codominantly expressed and can be used to measure mutation induction in most mammalian cells (5,6,19,27,90). Since the types of mutational damage, as well as the mechanisms by which they are produced, may vary considerably for different mutagens, specific genetic loci may differ markedly in their mutability by a particular agent. Mutagenesis assay systems employing multiple genetic markers may be more effective in detecting a broader spectrum of mutagenic and potentially carcinogenic agents. Extensive validation and application of the L5178Y $TK^{+/-}$ mouse lymphoma mutagenesis assay developed by Clive and co-workers (21-23,25, 26,67), which permits quantitation of forward mutation at the autosomal thymidine kinase (tk) locus, has revealed apparent differences in the mutagenic potential of certain agents at the hgprt and tk loci. Several chemicals that are only weakly or non-mutagenic at the hgprt locus appear to be strongly mutagenic at the tk locus (26). Operationally, the relatively short time required for phenotypic expression of induced mutations at the tk locus (10,26,67,85) facilitates its use in mutagenesis assays. Since technical disadvantages limit the utility of the $TG^r$ marker in the L5178Y $TK^{+/-}$ mouse lymphoma system (22,23,26,58,67), we were encouraged to develop a CHO multiple-marker mutagenesis assay system that could use both the hgprt and tk loci and would combine and expand the attributes of the two major in vitro mammalian-cell mutagenesis assay systems currently being used.

In this study, we describe the derivation of a CHO cell line that is heterozygous for both the adenine phosphoribosyltransferase (aprt) and thymidine kinase (tk) loci. This subline allows single-step selection of autosomal recessive $AA^r$ or $FUdR^r$ mutant phenotypes as well as the more commonly used $OUA^R$ or $TG^r$ genetic markers. Biochemical and genetic characterization of the heterozygous cell line, and dose response data for mutation induction at these four genetic loci by the direct-acting mutagen, ethyl methanesulfonate (EMS), are presented. We also establish optimal conditions for the phenotypic expression and selection of $AA^r$ and $FUdR^r$ mutants of CHO cells and present biochemical validation of the mutant phenotypes. Optimal drug concentrations, cell plating densities, and expression time requirements are determined for all four drug-resistance markers. Mutation data are reported for direct mutagens (EMS, MNNG, NQO) and promutagens requiring metabolic activation (DMN. BP). Finally, we discuss the role and expected use of the multiple-marker mutagensis assay to yield increased sensitivity and reliability in detecting genetic damage induced by complex pollutant mixtures of environmental concern.

## SECTION 2

## CONCLUSIONS AND RECOMMENDATIONS

The primary objective of this program has been to develop, validate, and apply multiple-marker, in vitro assays using mammalian cells to quantify the mutagenic effcts of pollutants arising from energy extraction, conversion, and use. The multiple-marker system, which was initially validated with data from standardized dose-response protocols, is now being applied to analyzing complex organic mixtures from crude effluents. Because most of the suspected mutagens associated with energy by-products and effluents require metabolic activation, activation techniques that can be coupled with the in vitro mammalian assay are being optimized. Experiments are being designed to compare the relative efficacy of organ homogenates (S9 or microsomes) versus cell-mediated activation and also to test whether our system will yield data at multiple gene loci which are proportional to the relative potency of compounds in other in vitro and in vivo systems. To further validate the system, paired compounds (carcinogenic and noncarcinogenic) have been tested. In this study (MRC/ICI/NIEHS, Mut. Res. 54: 203, 1978), results from the multiple marker assay are being compared with results from a wide assortment of test systems, including human cells. The simultaneous use of multiple markers is expected to test differences in sensitivity or mechanism of damage at the genetic loci involved, and such a system may increase the generality and reliability of mutagenesis assays for compounds having diverse interaction with mammalian genetic material.

Since the mutagenesis assay at mutiple gene loci may be a sensitive detector of genetic damage induced by complex pollutants of environmental concern, it should be applied to evaluating potential health effects associated with effluents from a wide variety of sources. To determine the versatility and general applicability of the multiple marker approach to studies of fractions from crude effluents, chemicals representative of many different classes should be tested. Such compounds, e.g., aromatic amines, alkyl halides, polycyclic aromatic hydrocarbons, nitrogen-containing heterocyclics, aliphatics, and aromatics, are frequently found in pollutant samples and have a broad spectrum of potentially hazardous interactions with mammalian DNA. Many problems are expected in exposing crude effluents to mammalian cells with an in vitro activation system (general toxicity, inhibitory or synergistic effects among mutagens, chemical transformation); in vitro mammalian bioassays must be adapted to accept crude mixtures and fractionated samples. These assays for forward mutation at several gene loci are expected to show a response to a broad variety of genetic damage,

minimizing false positive and false negative tests. The latter point is important because while many pure compounds have been tested, the frequency of false positive and negative results is not presently known for complex organic mixtures or effluent samples.

## SECTION 3

## MATERIALS AND METHODS

Cells and culture conditions. The parental cell line for these studies was a Chinese hamster ovary (CHO) cell line designated CHO-CIOA, obtained from Dr. W C. Dewey. One of the aprt $^{+/-}$ heterozygotes we derived from this line, CHO-IB-2, was subsequently used to derive sublines that were also heterozygous for the tk locus. One such subline, designated CHO-AT3-2, has been further characterized and used in a series of mutagenesis experiments described in this and succeeding papers (3,16; Adair & Carver, manuscript in preparation). Other Chinese hamster cell lines used in this study were the thymidine kinase-deficient strains DON-a3 (obtained from Dr. T.B. Shows), and RJK-92, originally designated 462-10 (obtained from Dr. R.G. Penwick).

Cells were routinely maintained at 37°C in α-MEM (K.C. Biological, Inc.) supplemented with antibiotics (penicillin/streptomycin) and 10% fetal calf serum (K.C. Biological, Inc.). Cell numbers were determined with a Coulter electronic particle counter. Cultures were periodically tested and confirmed to be free of mycoplasma. Under standard growth conditions, the doubling times ($T_2$) of our stock CHO-IB-2 and CHO-AT3-2 cell lines ranged from 14 to 16 h in monolayer culture and 14 to 18 h in suspension culture. The doubling time of CHO-IB-2 in suspension culture containing dialyzed fetal calf serum (DFCS) was often 20-22 h with substantial lag time after dilution; CHO-AT3-2 displayed a consistent doubling time under all culture conditions, with no significant delays after dilution.

Selection of presumptive heterozygotes for the aprt locus. Presumptive aprt $^{+/-}$ heterozygotes with intermediate levels of APRT activity were selected on the basis of their resistance to low concentrations of AA. Unmutagenized CHO-CIOA cells and cells from six clonal sublines of CHO-CIOA were plated at $10^5$-$10^6$/100 mm dish, in a series of selection media containing from 3.0 to 5.0 μg/ml AA. The selection media were changed after one week. Background growth of cells was excessive at the lower AA concentrations, but discrete colonies were obtained in most of the cultures at 4.5 to 5.0 μg/ml AA. Considerable clonal variation in the frequency of these low-level $AA^r$ colonies was observed. A large number of colonies were isolated, plated into fresh selection medium containing 5 μg/ml AA to test whether they were stably resistant, and then secured in liquid nitrogen.

Isolation of $HAT^r$ revertants of TK-deficient mutants. TK-deficient mutants of CHO-IB-2 were selected in 150 µg/ml BUdR. Cells from eight spontaneous $BUdR^r$ strains with barely-detectable levels of TK activity ($\leq$1% that of wild-type) were subsequently plated into HAT medium (α-MEM + FCS plus 100 µM hypoxanthine, 4 µM amethopterin, 50 µM thymidine), to select for revertants which had regained partial TK activity. Cells that lack TK activity are unable to utilize exogenous thymidine and are killed in the presence of amethopterin. Seven of the eight $tk^{-/-}$ strains yielded no spontaneous revertants in initial experiments (revertant frequencies $< 3 \times 10^{-7}$). To obtain $HAT^r$ revertants, monolayer cultures of each of the eight $tk^{-/-}$ strains were treated with EMS (150 µg/ml, 16 hours), subcultured at 2-day intervals for 8 days to allow fixation and phenotypic expression of induced mutations, and then plated into HAT selection medium ($5 \times 10^5$ cells/100-mm dish, 12 replicate dishes/culture). Five of the eight lines yielded $HAT^r$ colonies, at frequencies ranging from $3 \times 10^{-7}$ to $6 \times 10^{-5}$.

Preparation of liver homogenate (S-9 and microsomes). Eight week old outbred male rats (Simonsen albino, Sprague-Dawley derived) were injected i.p. with Aroclor 1254 (200 mg/ml in corn oil) at 500 mg/kg body weight. Four to five d later, they were sacrificed by immobilization in $CO_2$ atmosphere followed by arterial bleeding. Livers from $\geq$6 rats were dissected aseptically, washed with cold, sterile PBS, placed in PBS (3 mls PBS/ gm wet liver weight), and homogenized with a Polytron homogenizer (Brinkmann Instruments) at setting 4 for 1 m. The homogenate was centrifuged at 9000 x g for 15 m at 4°C and the supernatant (S-9) was quickly frozen and maintained at -80°C for $\leq$6 m. For the preparation of microsomes (µ), the S-9 supernatant was immediately centrifuged at 100,000 x g for 1 h. The pellet was gently homogenized, resuspended in sterile PBS + 10% glycerol, and maintained at -80°C. Protein concentration was determined by the method of Lowry (63).

Preparation of cell extracts. Aliquots of $2 \times 10^7$ cells were taken from suspension cultures of mutant or wild-type cells in exponential growth phase. Cells were collected by low-speed centrifugation, washed by resuspension in cold (0-4°C) phosphate-buffered saline (PBS), and recentrifuged. Each cell pellet was resuspended in 1.0 ml of cold extraction buffer (50 mM $KH_2PO_4$, 10 mM 2-mercaptoethanol, 1.0% Triton X-100, adjusted to pH 7), and the cell suspensions were sonicated on ice using a Branson model S125 ultrasonic cell disruptor with a microprobe tip. Cells were lysed with four 5-second bursts (power setting 4), with 10-second cooling intervals. Resulting cell lysates were cleared of debris by centrifugation for 30 minutes at 12,000 x g (0-4°C), and the supernatants were immediately frozen and stored at -80°C. Protein concentrations were determined by the method of Lowry *et al.* (63) using crystallized bovine serum albumin as a standard.

Adenine phosphoribosyltransferase assays. Adenine phosphoribosyltransferase (APRT) activities were assayed using the radioisotopic/DEAE filter disc method of Fenwick and Caskey (37), which measures the rate of conversion of $^{14}C$-adenine to $^{14}C$-AMP by APRT. Each 60 µl reaction mixture contained 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$,

2 mM 5-phosphorylribose-1-pyrophosphate (dimagnesium salt), 0.2 mM 8-$^{14}$C-adenine (60 Ci/mol), and 0-48 µg extract protein. All assays were run in duplicate. Background determinations were obtained by substituting extract buffer for cell extract. Reactions were initiated by adding cell extract to prewarmed reaction mixtures. Assay mixtures were incubated at 37°C, and 20 µl samples were taken after 15 and 30 minutes. Reactions were terminated by spotting the samples directly onto 2.4 cm DE-81 DEAE filter discs (Whatman) that had been pre-treated with 50 µl of 100 mM EDTA, pH 7.0. After the samples had dried, the discs were washed four times with 20 ml volumes of 1:1 methanol-$H_2O$, once with $H_2O$, and once with 95% ethanol, to remove unreacted $^{14}$C-adenine. The amount of $^{14}$C-AMP bound to the discs was subsequently counted by liquid scintillation. Reaction rates were constant with time for at least 30 minutes and proportional to the amount of extract protein in the reaction mixture over the range 0-48 µg protein. APRT activities of *aprt* $^{+/-}$ heterozygotes and AA$^r$ mutants have been normalized to the mean specific activity of wild-type CHO-C1OA cells, 4.3 nmol/min/mg protein.

Thymidine kinase assays. Thymidine kinase (TK) activities were assayed using the DEAE-disc method described by Roufa *et al.* (80), which measures the rate of phosphorylation of $^{14}$C-thymidine by TK. Each 50 µl reaction mixture contained 200 µM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 5 mM ATP, 40 µM 2-$^{14}$C-thymidine (58 Ci/mol), and 20-80 µg extract protein. All assays were run in duplicate. Background determinations were obtained by substituting extract buffer for cell extract. Reactions were initiated by adding cell extract to prewarmed reaction mixtures. Assay mixtures were incubated at 37°C for 10-20 minutes, and reactions were terminated by dilution with 3 ml of ice-cold 1 M sodium formate, pH 6.6. Diluted reaction mixtures were immediately filtered through 2.4 cm DE-81 DEAE filter discs (Whatman), which were rinsed three times with cold 1 mM sodium formate and allowed to dry before counting by liquid scintillation. Background counts represented less than 0.1% of the input radioactivity. TK activities of thymidine analog-resistant mutants and *tk* $^{+/-}$ heterozygotes have been normalized to the mean specific activity for CHO-IB-2 cells, 171$\pm$ 15 pmol/min/mg protein.

Chemicals and radioisotopes. All chemicals were obtained from Sigma or Calbiochem except DMN (Aldrich), 4-NQO (K & K Laboratories), BP (Eastman), 5-phosphorylribose-1-pyrophosphate (P.L. Biochemicals), and polyethylene glycol 1000 or PEG (J.T. Baker Chemicals Co.). All were stored at $\leq 4°C$. The mutagens EMS and DMN (aqueous), MNNG (in ethanol), or NQO (in DMSO, final [DMSO] $\leq$0.015%) were prepared and diluted with growth medium immediately before use. The BP stock (in DMSO) was stored $\leq$ 6 months at room temperature; the stock was not filter-sterilized. Purine analogues (AA, AG, TG) were dissolved in NaOH and adjusted to pH 7.4 with HCl; ouabain was dissolved in 100°C $H_2O$ and immediately diluted with growth medium. The BUdR, FUdR, and TFT stock solutions (aqueous) were stored frozen at -20°C and protected from light during all procedures. All solutions (except BP) were sterilized by filtration through a 0.22 µm pore filter (Millipore Corp.) before use. Radioisotopes (8-14C-adenine and 2-14C-Thymidine) were obtained from Amersham.

Statistics. Where shown, error bars represent one standard error of the mean (SEM). To determine the extent of variance from the mean, both the standard deviation of the data points (experimental error) and the counting error (sampling or Poisson error) were calculated; in all cases, the larger of these values was used to determine the standard error (7). Straight line fits were made by standard linear regression analysis. The t test to obtain P values used Scheffe's special d-solution to the Behrens-Fisher problem (81); for degrees of freedom, a conservative and minimal value (n-2) was used, with n equal to the number of data points in the data set with the smallest number of observations.

## SECTION 4
## EXPERIMENTAL PROCEDURES

Mutagenesis. Approximately 18-24 h before treatment with mutagens, cells from a recently-thawed culture were plated at 1 to 2 x $10^6$ per T25 flask or 3 to 4 x $10^6$ per T75 flask (Corning). Treatment with direct mutagens was carried out in medium supplemented with FCS (α-MEM FCS) by exposing monolayer cultures to fresh medium or fresh medium containing the mutagen ethyl methanesulfonate (EMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), or 4-nitroquinolinel-oxide (NQO). After 16-18 h the mutagen-containing medium was removed, the cells were rinsed and dispersed with trypsin, and 3 to 5 x $10^6$ cells were placed into suspension culture for expression of phenotypes. Treatment with promutagens requiring metabolic activation involved a 2 h exposure in α-MEM (serum-free) containing S-9 or microsomes plus the following co-factors: NADPH, 0.37 mM; NADH, 0.93 mM; NADP, 0.87 mM; glucose-6-phosphate, 6.57 mM. Following exposure, the cells were washed and allowed a 1 h recovery period in α-MEM FCS. Approximately 1 to 2 x $10^6$ cells were carried forward in monolayer culture for expression. After three days incubation, 3 to 5 x $10^6$ cells were place into suspension culture for continued phenotypic expression (up to 10 d) and subsequent replating for mutant selection.

After mutagen treatment, aliquots of cells were plated at 200 to 3000 cells/60-mm dish (six replicates per plating) to determine the surviving cell fraction ($\bar{S}$). Following 7 d of incubation, surviving colonies were fixed with methanol, stained with 0.5% crystal violet, and those colonies containing 100 or more cells were counted. The plating efficiencies of untreated controls in α-MEM FCS ranged from 0.65 to 0.90; in medium supplemented with extensively dialyzed FCS (α-MEM DFCS, K.C. Biological, Inc), plating efficiencies ranged from 0.55 to 0.75.

Mutant Selection. To quantitate mutation induction by the replating method, mutagenized or untreated (control) cell populations were incubated in monolayer and/or suspension culture to allow expression of mutant phenotypes. Cell populations treated with direct mutagens were maintained for 7-10 d in suspension culture; those exposed to promutagens requiring metabolic activation were maintained for the first three d in monolayer culture and subsequently transferred to suspension culture for 4-7 d additional expression time. After expression, the cells were resuspended, plated, and incubated as follows:

| Mutant Phenotype | Concentration of Selecting Drug (μg/ml) | # Cells Plated per 100-mm petri dish | # Days Incubation at 37° |
|---|---|---|---|
| $AA^r$ | 80 (0.59 mM) | $3 \times 10^5$ | 12-14 |
| $AG^r$ | 40 (0.26 mM) | $3 \times 10^5$ | 16-18 |
| $TG^r$ | 4 (0.024 mM) | $2 \times 10^5$ | 12-14 |
| $OUA^R$ | 2200 (3.00 mM) | $8 \times 10^5$ | 10-12 |
| $FUdR^r$ | 2 (0.008 mM) | $3 \times 10^4$ | 12-14 |
| $BUDR^r$ | 150 (0.49 mM) | $3 \times 10^4$ | 12-12 |
| $TFT^r$ | 3 (0.01 mM) | $3 \times 10^4$ | 12-14 |

Twelve to twenty-four replicate 100-mm dishes containing 16-20 ml of drug-selection medium were plated for each selecting drug.

With the CHO-AT3-2 cell line, the colony size in extensively dialyzed serum was enhanced by the addition of deoxycytidine at 10-20 μg/ml (0.076 mM). Media containing DFCS was used for mutant selection with AA, TG, and FUdR and included deoxycytidine at 20 μg/ml; FUdR selection also included uridine at 10 μg/ml (0.04 mM) to reduce incorporation of FUdR metabolites into RNA (42,43).

To quantitate mutation induction by the in situ method, mutagenized cells were plated in 100-mm petri dishes at approximately $1.2 \times 10^4$ cells/dish and incubated until the number of cells had increased to the equivalent of five to six doublings; at that time, the medium was carefully removed and fresh medium containing the appropriate selecting drug was added. The relative increase in the number of cells during the expression period was monitored by counting (after dispersing with trypsin) replicate cultures that had been inoculated at the same density as in the mutation experiment. The increase was also determined by plating 100-mm dishes with approximately $4 \times 10^3$ cells in 100-mm dishes and counting the number of cells per colony-forming group at appropriate intervals (13). The maximum density of cells in the control was about 4 to $8 \times 10^5$ cells per 100-mm dish (i.e., approximately 0.75 to $1.5 \times 10^4$ cells/$cm^2$ or $\sim 10^4$ colonies). The actual cell density in mutagenized cultures was somewhat lower and dependent upon the dose of mutagen.

To determine the plating efficiency ($\overline{PE}$) at the time of mutant selection, 200 to 400 cells plated in 60-mm dishes (six replicates per condition) were counted. The observed mutant frequency represents the mean number of mutant colonies per dish divided by the number of viable cells, i.e., the number of cells plated per dish, corrected for $\overline{PE}$. The induced

mutant frequency is the difference between the observed mutant frequency of treated cultures and the observed spontaneous mutant frequency of controls.

Luria-Delbruck fluctuation analysis. The pattern and rate of appearance of drug-resistant variants in cultures was determined by fluctuation analysis (11,62,64). Thirty-two independent replicate cultures were initiated from inocula of 50 or 100 cells. The cultures were grown for 9-10 days (approximately 14-16 doublings) and plated into medium containing the appropriate selection drug. The distribution of drug-resistant colonies was analyzed by the methods of Luria and Delbruck (64) and Lea and Coulson (62) to obtain estimates of the apparent spontaneous mutation rates for the *aprt* and *tk* loci in CHO-IB-2 or CHO-AT3-2 cells.

Cell hybridization. Cell hybrids were obtained by PEG-induced fusion, using the procedure of Davidson and coworkers (33) as modified by Adair *et al.* (4). PEG-treated cultures were incubated overnight in α-MEM + FCS, then trypsinized and replated into HAT medium to select for complementing cell hybrids. Cells that lack TK activity are unable to utilize exogenous thymidine and are killed in the presence of amethopterin. Complementation in cell hybrids between two thymidine analog-resistant mutants would allow colony formation in HAT selection medium, and would indicate that the two mutants did not share the same genetic defect. Cell hybrids between HGPRT-deficient $TG^r$ cells and each thymidine analog-resistant strain were included in complementation analysis experiments to determine whether the TK-deficient mutant phenotypes were dominant or recessive, and as a positive control for complementation. Controls for reversion for each mutant strain were also included, but no revertants were observed in complementation analysis experiments.

Segregation analysis. Utilizing one of the TK-deficient, $BUdR^r$ strains we had previously isolated from CHO-IB-2, we selected a spontaneous $AA^r$ mutant that lacked both APRT and TK activities. Cells from this *aprt* $^{-/-}$, *tk* $^{-/-}$ strain were subsequently hybridized by PEG-induced fusion with a $TG^r$ subline of CHO-AT3-2 or $TG^r$ subline of wild-type (*tk* $^{+/+}$) CHO, to test whether CHO-AT3-2 cells were indeed functionally hemizygous at both the *aprt* and *tk* loci. Hybrids were selected and grown to mass culture in HAT medium. An inoculum of $3 \times 10^4$ $HAT^r$ cells of each hybrid were then grown for approximately ten generations in the absence of further selection and plated into medium containing either 80 μg/ml AA or 150 μg/ml BUdR. Spontaneous nondisjunction involving the segregation or loss of dominant wild-type alleles for the *aprt* or *tk* loci from such hybrids will result in reexpression of recessive $AA^r$ or $BUdR^r$ phenotypes. The frequencies of such drug-resistant segregants should be much higher in hybrids between mutant X heterozygotes than in hybrids between mutant X wild-type cells; only one wild-type allele would have to be lost for reexpression of the mutant phenotype in the former case, while two wild-type alleles would have to be lost in the latter. Thus, by comparing the drug-resistant segregant frequencies in the above hybrids, we could determine whether CHO-AT3-2 cells were indeed heterozygous at both the *aprt* and *tk* loci.

## SECTION 5
## RESULTS

Isolation and characterization of aprt$^{+/-}$ heterozygotes. Presumptive heterozygotes for the autosomal aprt locus were selected from unmutagenized populations of CHO-CIOA or clonal sublines, on the basis of resistance to low levels of AA (Fig. 1). This approach was originally used by Jones and Sargent (52) to isolate aprt heterozygotes from a different CHO strain, but a subsequent attempt to isolate heterozygotes using similar approaches was unsuccessful (34). As reported by Jones and Sargent, resistance to AA is correlated with a loss of APRT activity and occurs in two discrete steps. The concentration of AA necessary to reduce CHO-CIOA survival to 10% ($D_{10}$) was approximately 2.5 µg/ml, compared to 7-11 µg/ml reported by Jones and Sargent. Using concentrations of 4-5 µg/ml AA, which reduced CHO-CIOA survival to approximately $10^{-4}$, we were able to obtain aprt $^{+/-}$ heterozygotes with intermediate levels of APRT activity (Fig. 1). Eleven out of twelve presumptive heterozygotes, confirmed to be stably resistant to 5 µg/ml AA and subsequently assayed for APRT activity, had specific activities ranging from 47% to 78% that of wild-type CHO-CIOA. Three of these presumptive heterozygotes (designated IIA-1, IB-2, IID-4) with approximately 50% wild-type APRT activity were subsequently employed in second step selections for spontaneous and EMS-induced mutants resistant to higher levels of AA (Fig. 1). Mutants resistant to 20-80 µg/ml AA were isolated and assayed for APRT activity. Fifty-five of the fifty-nine $AA^r$ colonies assayed (93%) had ≤ 12% wild-type APRT activity. No significant differences in residual APRT activity were observed for mutants selected at 20, 40, or 80 µg/ml AA. On the basis of its favorable growth and plating chacteristics, one aprt $^{+/-}$ strain, CHO-IB-2, was chosen for further genetic analysis and use in mutagenesis experiments.

Forward mutation at the aprt locus. Spontaneous and EMS-induced forward mutation frequencies for the aprt locus for wild-type CHO-CIOA-19 and the aprt $^{+/-}$ heterozygote, CHO-IB-2, are shown in Table 1. Observed frequencies of mutants resistant to 80 µg/ml AA were at least several orders of magnitude higher for the aprt $^{+/-}$ heterozygote than for wild-type, aprt $^{+/+}$ cells. High frequencies of $AA^r$ mutants were induced by treating CHO-IB-2 cells with known direct-acting mutagens such as EMS (Table 1), indicating that this subline and the $AA^r$ marker would be practical for mutagenesis assays. Most of the $AA^r$ mutants assayed had ≤ 5% wild-type APRT activity. The spontaneous forward mutation rate at the aprt locus in CHO-IB-2 cells was determined by Luria-Delbruck fluctuation analysis to be approximately $3 \times 10^{-7}$ per cell per generation (Table 2). This value agrees closely with the rate reported by Jones and Sargent (52).

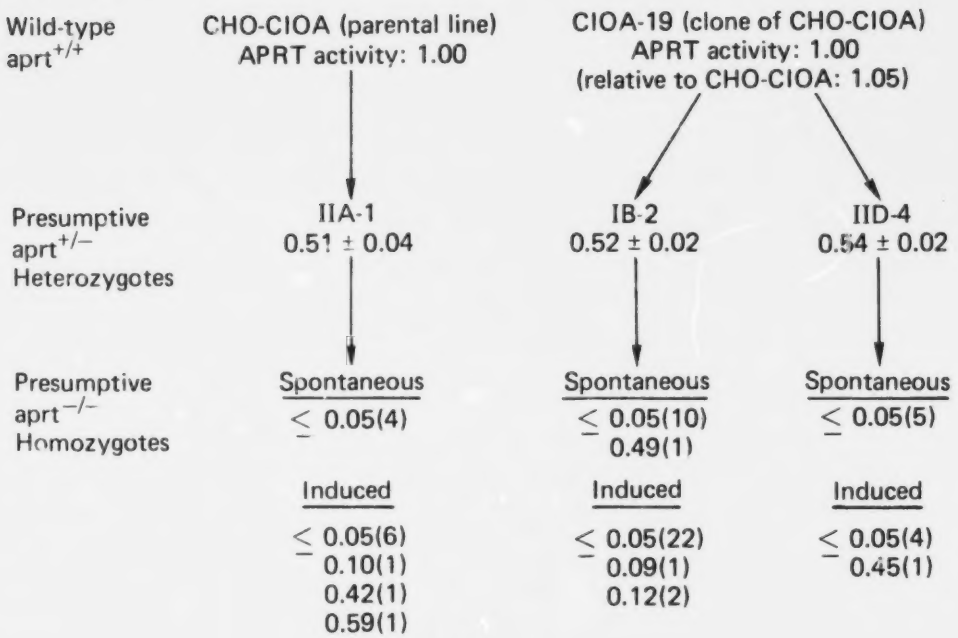



Figure 1. Derivation of aprt$^{+/-}$ heterozygous cell lines, with subsequent single-step selection of spontaneous and EMS-induced mutants resistant to AA (aprt$^{-/-}$). Relative APRT activities are shown, with the number of clones tested in parentheses.

Derivation of heterozygotes for the tk locus. Although aprt $^{+/-}$ heterozygotes had been readily selected on the basis of their resistance to intermediate levels of AA, a similar approach for isolating tk $^{+/-}$ heterozygotes was unsuccessful. Direct, single-step selection of tk $^{+/-}$ heterozygotes in intermediate concentrations of BUdR was impractical due to excessive background growth of wild-type cells. Attempts to isolate tk $^{+/-}$ heterozygotes using another thymidine analog, TFT, yielded unstable drug-resistant variants at high spontaneous frequencies (2). Thus, we were forced to employ an indirect approach similar to that of Clive et al. (22), first isolating rare tk $^{-/-}$ mutants of CHO-IB-2 on the basis of their resistance to high levels of BUdR, and subsequently selecting HAT$^r$ revertants of those cells (Fig. 2). We recovered BUdR$^r$ colonies from unmutagenized CHO-IB-2 cell populations in three of five selection experiments, at an overall frequency of $2 \times 10^{-7}$ (Table 1). All eight spontaneous BUdR$^r$ strains assayed, as well as 32/32 mutagen-induced BUdR$^r$ or FUdR$^r$ isolates, had barely detectable levels of TK activity ( $\leq 1\%$ that of wild-type). All of these tk $^{-/-}$ mutants were stable, HAT-sensitive, and cross-resistant to BUdR, FUdR, and TFT. When 8 tk $^{-/-}$ mutants were subjected to EMS mutagenesis and plated into HAT medium, five of the eight strains yielded HAT$^r$ colonies, at frequencies ranging from $3 \times 10^{-7}$ to $6 \times 10^{-5}$. To determine whether

Table 1

Forward mutation frequencies of parental and heterozygous cell lines

| Cell line | Selecting Drug | Spontaneous | EMS-induced[a] |
|---|---|---|---|
| CHO-C1OA-19 | AA | $3.0 \times 10^{-7}$ | $9.6 \pm 5.2 \times 10^{-6}$[b] |
| parental line | BUdR | $9.0 \pm 6.4 \times 10^{-7}$ | $1.4 \pm 0.3 \times 10^{-5}$[b] |
| (*aprt* $^{+/+}$, *tk* $^{+/+}$) | FUdR | $5.0 \times 10^{-7}$ | $3.7 \pm 1.3 \times 10^{-6}$[b] |
| IB-2 | AA | $3.7 \pm 0.4 \times 10^{-5}$ | $6.6 \pm 0.5 \times 10^{-4}$[b] |
| aprt heterozygote | BUdR | $2.4 \pm 0.8 \times 10^{-7}$ | $2.3 \pm 1.0 \times 10^{-6}$[c] |
| (*aprt* $^{+/-}$, *tk* $^{+/+}$) | FUdR | $7.0 \pm 7.0 \times 10^{-8}$ | $5.0 \pm 5.0 \times 10^{-7}$[c] |
| AT3-2 | AA | $2.7 \pm 0.2 \times 10^{-5}$ | $1.5 \pm 0.1 \times 10^{-3}$[d] |
| aprt, tk heterozygote | BUdR | $2.8 \pm 0.2 \times 10^{-3}$ | - |
| (*aprt* $^{+/-}$, *tk* $^{+/-}$) | FUdR | $1.6 \pm 0.2 \times 10^{-3}$ | $6.1 \pm 0.3 \times 10^{-3}$[d] |

[a] Expression time 7-8d.

[b] 100-125 μg/ml, 16-18 hr exposure, $\bar{S} \sim 0.65 - 0.70$.

[c] 160 μg/ml, 16 hr exposure, $\bar{S} \sim 0.35$.

[d] 420 μg/ml, 16 hr exposure, $\bar{S} \sim 0.30$.

Table 2

Luria-Delbruck fluctuation analysis of

azaadenine-resistance in IB-2 cells

| | | |
|---|---|---|
| Replicate cultures[a] | 32 | 1 |
| Initial number of cells per replicate | 100 | 100 |
| Final number of cells per replicate | $1.75 \times 10^7$ | $1.75 \times 10^7$ |
| Cells tested per sample[b]; (# samples) | $3.6 \times 10^6$ (1) | $3 \times 10^5$ (48) |
| Number of mutants per sample: | | |
| Range | 0-89 | 0-2 |
| Median | 1 | 0 |
| Mean | 3.7 | 0.31[c] |
| Variance | 244 | 0.38[c] |
| Ratio: Variance/Mean | 65.5 | 1.23[c] |
| Null fraction | 0.47 | 0.85 |
| Mutation rate calculated by: | | |
| Mean method[d] | $3.6 \times 10^{-7}$ | |
| Median method[e] | $3.2 \times 10^{-7}$ | |
| Null fraction method[e] | $2.7 \pm 0.6 \times 10^{-7}$ | |
| Maximum likelihood method[e] | $2.4 \pm 0.5 \times 10^{-7}$ | |

[a]Replicate cultures initiated by innoculating Corning T-25 flasks with 100 cells were subsequently transfered to T-75 flasks.

[b]Cells were plated at $3 \times 10^5$/100-mm dish in medium containing 80 µg/ml AA and incubated for 12 d to allow colony formation.

[c]These numbers may be underestimated due to limitations of sample size which resulted in many plates with no mutant colonies.

[d]Ref. 11.

[e]Ref. 62.

these colonies actually represented tk $^{+/-}$ revertants, a total of 33 HAT$^{r}$ colonies were isolated, grown to mass culture, and assayed for TK activity. TK speciric activities ranged from 7-184 pmol/min/mg protein (compared to 171 ± 15 pmol/min/mg protein for wild-type CHO-IB-2), with most of the revertants having 10-30% wild-type TK activity. Further biochemical characterization of several of these presumptive tk $^{+/-}$ heterozygotes (Adair, manuscript in preparation) revealed differences in in vitro thermolability and substrate concentration optima for TK, suggesting that they indeed represent structural gene mutations. One of these tk $^{+/-}$ heterozygotes, CHO-AT3-2, was chosen for further genetic analysis and use in mutagenesis assays.

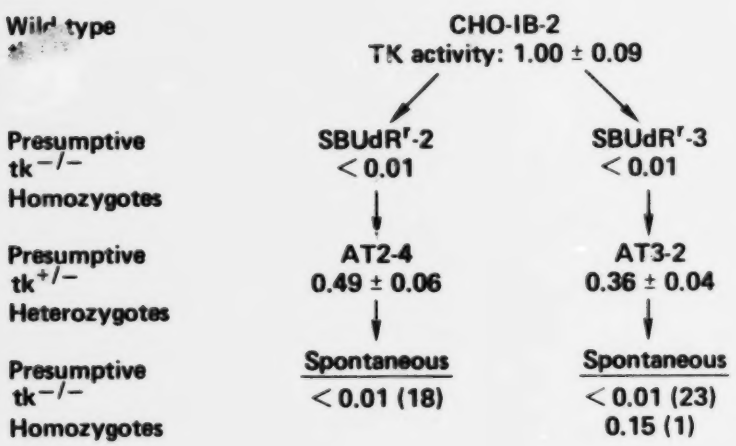



Figure 2. Derivation of tk$^{+/-}$ heterozygous cell lines by isolating tk$^{-/-}$ mutants of CHO-IB-2, with subsequent EMS mutagenesis and HAT selection of tk$^{+/-}$ revertants. Spontaneous mutants resistant to FUdR were recovered in a single step; relative TK activities are shown, with the number of clones tested in parentheses.

Forward mutation at the tk locus. Although BUdR$^r$ or FUdR$^r$, tk $^{-/-}$ mutants were rarely recovered from unmutagenized CHO-IB-2 populations (Table 1), such variants were readily obtained from CHO-AT3-2 at very high spontaneous frequencies (2-3 x $10^{-3}$). To determine whether expression of tk $^{-/-}$, thymidine analog-resistant phenotypes in CHO-AT3-2 indeed represented spontaneous, randomly occurring "mutational" events, and whether the high frequencies of these variants in unmutagenized populations reflected a high spontaneous rate of mutation at the tk locus, we performed a Luria-Delbruck fluctuation analysis experiment. Cells from each replicate culture were plated into media containing BUdR, FUdR or TFT, allowing us to determine the spontaneous rate and pattern of occurrence of drug-resistance for each selection agent. In each case, as shown by data in Table 3, the variance for replicate, independent cultures was significantly greater than that for multiple platings of a single culture. These results indicate that tk $^{-/-}$, thymidine analog-resistant variants arose spontaneously and randomly in CHO-AT3-2 populations, suggesting that acquisition of the thymidine analog-resistant phenotype represented a "mutational" event occurring prior to drug selection and was not merely an adaptive response to the selecting drug. Calculation of apparent forward mutation rates by several methods (Table 3) indicated that tk $^{-/-}$, thymidine analog-resistant variants arise at a rate of 2-3 x $10^{-4}$ per cell per generation. These estimated mutation rates are approximately three orders of magnitude higher than those for single-step, forward mutation at the hgprt or aprt loci in the same cell line (Table 2 and unpublished results), and are simlar for BUdR, FUdR, and TFT selections.

Complementation analysis of thymidine analog-resistant strains. The thymidine analog-resistant strains we have isolated characteristically lack appreciable TK activity and thus are killed in HAT selection medium. To determine whether the HAT-sensitive phenotypes of these mutants were dominant or recessive, and to determine whether all shared the same genetic deficiency. a series of mutants were subjected to complementation analysis by fusion in pairwise crosses. Complementation analysis of BUdR$^r$, FUdR$^r$, and TFT$^r$ derivatives of CHO-AT3-2 with other TK-deficient mutant strains (Table 4), indicated that all of these mutants behave recessively and fail to complement each other or other tk $^{-/-}$ Chinese hamster cell lines such as DON-a3 or RJK-92 (V-79 462-10). Thus, all of these mutants belong to a single complementation class and presumably reflect mutational lesions at the same genetic locus. The DON-a3 cell line has been previously used to map this presumptive, structural gene locus for TK in both the mouse (60) and human (36) karyotypes.

Segregation analysis of heterozygosity in CHO-AT3-2. Further evidence that CHO-AT3-2 cells were indeed heterozygous at both the aprt and tk loci was obtained by comparing the frequencies of AA$^r$ or BUdR$^r$ segregants from mutant X CHO-AT3-2 and mutant X wild-type cell hybrids. For both loci, the frequencies of drug-resistant segregants from mutant X CHO-AT3-2 hybrids were at least 2-3 orders of magnitude greater than those from mutant X wild-type cells (Table 5), as would be expected in the case of a double heterozygote with a single wild-type allele at both the aprt and tk loci.

Table 3

Luria-Delbruck fluctuation analysis of thymidine analogue resistance in AT3-2 cells

| | | | | | | |
|---|---|---|---|---|---|---|
| Replicate cultures | 31 | 1 | | | | |
| Initial cell number per replicate | 50 | 50 | | | | |
| Final cell number per replicate | $2.7 \times 10^7$ | $2.7 \times 10^7$ | | | | |
| Cells tested per sample (# samples) | $9.0 \times 10^4$(1) | $9.0 \times 10^4$(12) | | | | |
| Number of mutants per sample: | | | | | | |
| | BUdR$^r$ | | FUdR$^r$ | | TFT$^r$ | |
| Range | 138-293 | 144-210 | 83-225 | 111-150 | 77-205 | 99-141 |
| Median | 180 | 174 | 124 | 120 | 127 | 117 |
| Mean | 188.6 | 173.1 | 134.6 | 40.9 | 122.7 | 120.0 |
| Variance | 1597 | 254 | 1266 | 321 | 852 | 206 |
| Ratio: Variance/Mean | 8.47 | 1.47 | 9.41 | 2.61 | 6.53 | 1.71 |
| Mutation rate calculated by: | | | | | | |
| Mean method[a] | $2.1 \pm 0.5 \times 10^{-4}$/cell/gen | | $1.6 \pm 0.4 \times 10^{-4}$/cell/gen | | $1.5 \pm 0.3 \times 10^{-4}$/cell/gen | |
| Median method[b] | $3.5 \pm 0.4 \times 10^{-4}$/cell/gen | | $2.6 \pm 0.3 \times 10^{-4}$/cell/gen | | $2.6 \pm 0.3 \times 10^{-4}$/cell/gen | |
| Accumulation with time[c], exp A | $2.7 \times 10^{-4}$/cell/gen | | $1.8 \times 10^{-4}$/cell/gen | | $1.8 \times 10^{-4}$/cell/gen | |
| exp B | $1.7 \times 10^{-4}$/cell/gen | | $1.2 \times 10^{-4}$/cell/gen | | $1.2 \times 10^{-4}$/cell/gen | |

[a]Ref. 64.

[b]Ref. 62.

[c]Ref. 82. Experiment A and B separated by three additional days of expression time.

Table 4

Complementation analysis of thymidine analog-resistant strains[a]

| | DON-a3[b] | RJK-92[c] | SBUdR$^r$-1 | SBUdR$^r$-2 | SBUdR$^r$-3 | ETFT$^r$-2 | BUdR$^r$ AT3-2 | FUdR$^r$ AT3-2 | TFT$^r$ AT3-2 | STG$^r$-1[d] |
|---|---|---|---|---|---|---|---|---|---|---|
| DON-a3 | - | | | | | | | | | |
| RJK-92 | - | - | | | | | | | | |
| SBUdR$^r$-1 | - | - | - | | | | | | | |
| SBUdR$^r$-2 | - | - | - | - | | | | | | |
| SBUdR$^r$-3 | - | - | - | - | - | | | | | |
| ETFT$^r$-2 | - | - | - | - | - | - | | | | |
| BUdR$^r$ AT3-2 | - | - | - | - | - | - | - | | | |
| FUdR$^r$ AT3-2 | - | - | - | - | - | - | - | - | | |
| TFT$^r$ AT3-2 | - | - | - | - | - | - | - | - | - | |
| STG$^r$-1 | + | + | + | + | + | + | + | + | + | - |

[a]Complementation (+) or noncomplementation (-) based upon growth of hybrids in HAT selection medium.

[b]TK-deficient strain of DON cells, isolated by Westerveld et al. (98).

[c]TK-deficient strain of V-79 cells (originally designated 462-10), characterized by Roufa et al. (80).

[d]HGPRT-deficient strain of CHO isolated on the basis of its resistance to 4 μg/ml TG.

Table 5

Segregation analysis of heterozygosity in CHO-AT3-2

| Hybrid | Frequency of drug-resistant segregant colonies[a] | |
|---|---|---|
| | $AA^r$ | $BUdR^r$ |
| $AA^r$, $BUdR^r$ Mutant X Wild-type CHO ($aprt^{-/-}$, $tk^{-/-}$) ($aprt^{+/+}$, $tk^{+/+}$) | | |
| Expt. 1 | $4.8 \pm 1.5 \times 10^{-6}$ | $4.3 \pm 4.3 \times 10^{-7}$ |
| Expt. 2 | $7.2 \pm 1.7 \times 10^{-6}$ | $13.4 \pm 7.7 \times 10^{-7}$ |
| $AA^r$, $BUdR^r$ Mutant X CHO-AT3-2 ($aprt^{-/-}$, $tk^{-/-}$) ($aprt^{+/-}$, $tk^{+/-}$) | | |
| Expt. 1 | $1.4 \pm 0.1 \times 10^{-3}$ | $2.5 \pm 0.5 \times 10^{-4}$ |
| Expt. 2 | $0.7 \pm 0.1 \times 10^{-3}$ | $5.0 \pm 0.7 \times 10^{-4}$ |

[a] Spontaneous frequencies determined after ~10 population doublings, by plating hybrid cells into media containing the appropriate selecting drug. Segregant frequencies have been corrected for $\overline{P.E.}$ (0.50-0.65).

Assay of specific locus mutation using multiple markers. Using CHO-AT3-2 cells, it is possible to quantitate mutation induction at the autosomal aprt and tk loci, as well as in the genes involved in OUA-resistance or at the X-linked hgprt locus. We have defined optimal conditions for the selection of $TG^r$, $AA^r$, $OUA^R$, and $BUdR^r$ or $FUdR^r$ mutant phenotypes. Establishment of selection parameters such as maximal cell plating densities and expression kinetics for each drug selection marker are described (16). Maximal $TG^r$ mutant frequencies are attained by 6-8 days of expression and plateau at that level. Full expression of $AA^r$ or $OUA^R$ mutants requires only 2-4 days, with no apparent loss of mutant cells by longer expression times. Expression kinetics for resistance to FUdR are more complex with mutagen-specific differences in optimal expression time (16; Adair & Carver, manuscript in preparation). Dose response data for mutation induction after 8 d expression by the direct-acting mutagen, EMS, in the genes for APRT, TK, HGPRT, and $Na^+/K^+$ ATPase ($OUA^R$) in CHO-AT3-2 cells are presented in Fig. 3. Significant dose-dependent increases in mutation frequency were observed for all four drug-resistance markers; in each case there is a linear dose-response relationship between mutation frequency and EMS concentration. The slopes for mutation induction as a function of applied molar dose were: $FUdR^r$, $1.31 \pm 0.04 \times 10^{-3}$/mM; $AA^r$, $4.14 \pm 0.05 \times 10^{-4}$/mM; $TG^r$, $5.21 \pm 0.14 \times 10^{-4}$/mM; $OUA^R$, $1.27 \pm 0.09 \times 10^{-4}$/mM. These data indicate that the gene for TK may be much more mutable than the genes for APRT, HGPRT, or $Na^+/K^+$ ATPase ($OUA^R$).

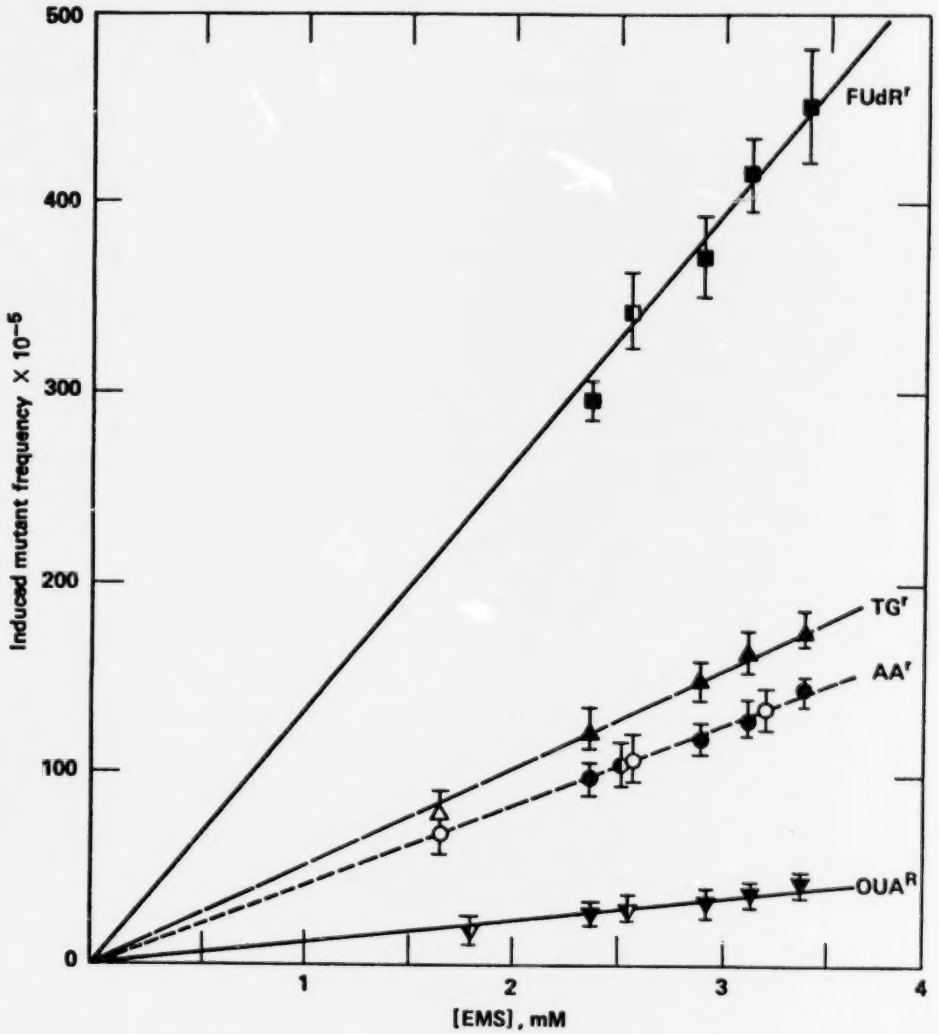



Figure 3. Increase in induced frequency of $FUdR^r$ (-■-), $TG^r$(- -▲- -)$AA^r$ (-●-), and $OUA^R$ (-▼-) mutants with increasing concentration (mM) of EMS. Different experiments are represented by open and closed symbols. The linear regression of the straight line fit ($Y = b_0 + b_1 X$, where X= EMS , mM) has the following parameters (± 1 S.D.):

$FUdR^r$: $b_0 = -0.04 \pm 0.11 \times 10^{-3}$; $b_1 = 1.31 \pm 0.04 \times 10^{-3}$

$TG^r$: $b_0 = -2.96 \pm 2.47 \times 10^{-5}$; $b_1 = 5.21 \pm 0.14 \times 10^{-4}$

$AA^r$: $b_0 = 0.02 \pm 1.37 \times 10^{-5}$; $b_1 = 4.14 \pm 0.05 \times 10^{-4}$

$OUA^R$: $b_0 = -1.65 \pm 2.38 \times 10^{-5}$; $b_1 = 1.27 \pm 0.09 \times 10^{-4}$

Cell Density Analysis. The number of wild type cells plated affects the survival of drug-resistant mutant cells in the selecting drug. As previously reported (13), reconstruction experiments with wild type CHO cells showed that a critical density for $AG^r$ mutant recovery in the selecting drug could be as low as 3 to 4 x $10^4$ cells/$cm^2$. For CHO-IB-2, a similar reconstruction experiment (Fig. 4) indicated that the mean critical density ($MCD_{50}$, or density of cells yielding 50% mutant survival) for recovery of an aprt $^{-/-}$, hgprt$^-$ mutant was approximately 1.3 x $10^4$ cells/$cm^2$ in 80 µg/ml AA and 3.0 x $10^4$ cells/$cm^2$ in 40 µg/ml AG. The recovery of two such spontaneous aprt$^{-/-}$, hgprt$^-$ mutants was reduced by 50% at approximately 7.5 x $10^3$ cells/$cm^2$ in 4 µg/ml TG.

Reconstruction experiments such as these measure the response of a single mutant phenotype, i.e., one prototype mutant clone isolated from a selection experiment and grown to a population sufficiently large for testing. However, a number of independent mutational events, and thus a variety of mutant phenotypes, contribute to the frequency of spontaneous mutants. In Fig. 5, an experiment designed to test the optimal plating density for maximum recovery of spontaneous mutants is shown. The observed spontaneous mutant frequencies of $AA^r$, $AG^r$, $TG^r$, $OUA^R$ and $FUdR^r/BUdR^r/TFT^r$ are compared as a function of the number of CHO-AT3-2 or CHO-IB-2 cells plated. The cell density ranged from 1 x $10^5$ to 1 x $10^6$ cells per plate (1 x $10^4$ to 1 x $10^5$ cells per plate for pyrimidine analogs), corresponding to approximately 1.9 x $10^3$ to 1.9 x $10^4$ cells/$cm^2$ and 1.9 x $10^2$ to 1.9 x $10^3$ cells/$cm^2$, respectively.

In Fig. 5a, recovery of $AA^r$ mutants decreased with increasing cell density (50% decrease at about 6.5 x $10^5$ cells/plate) corresponding to the metabolic cooperation observed in Fig. 4. The experiments described (Table 6, Figs. 8, 11a) used 80 µg/ml AA and 3 x $10^5$ cells/plate, a drug concentration and cell density that allows maximal recovery of spontaneous mutants. As shown in Fig. 5a, selection with either 30 or 40 µg/ml AG was not affected by cell concentrations up to 1 x $10^6$ cells/plate (1.85 x $10^4$ cells/$cm^2$). The dose response data described in Table 6 were obtained with 40 µg/ml AG and 3 x $10^5$ CHO-IB-2 cells/plate.

For both CHO-AT3-2 and CHO-IB-2, the optimal cell density for recovery of $TG^r$ mutants (Fig. 5b) is approximately 1 to 2 x $10^5$ cells/plate; mutant recovery was reduced by 50% at 8 to 9 x $10^3$ cells/$cm^2$. No significant increase in recovery was seen at either 5 x $10^4$ or 7.5 x $10^4$ cells/plate (data not shown). The data in Table 6 and Figs. 9 and 11b correspond to selection with 4 µg/ml TG and 2 x $10^5$ cells/plate. For $OUA^R$ mutants, no density effect was observed up to 8 x $10^5$ CHO-IB-2 cells/plate (Fig. 5b). This was tested not only with new spontaneous mutants but also in a reconstruction experiment with 200 $OUA^R$ cells/plate; the CHO-IB-2 cell density ranged from 1 x $10^5$ to 4 x $10^6$ cells per plate. The resulting plating efficiency (data not shown) showed no significant change as a function of the number of CHO-IB-2 cells, although smaller colonies were observed when 2 and 4 x $10^6$ cells were plated. The frequencies of $OUA^R$ mutants shown in Table 6, Figs. 10c and 11c correspond to selection with 3mM OUA and 8 x $10^5$ cells/plate.

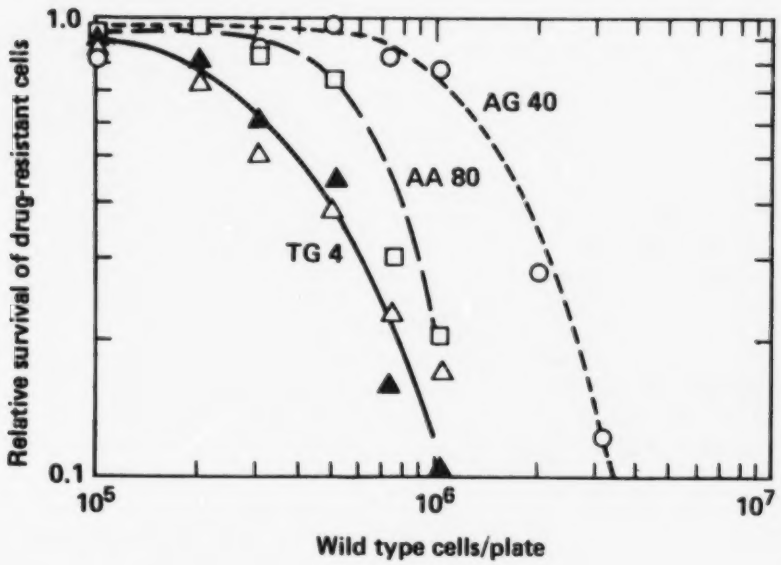
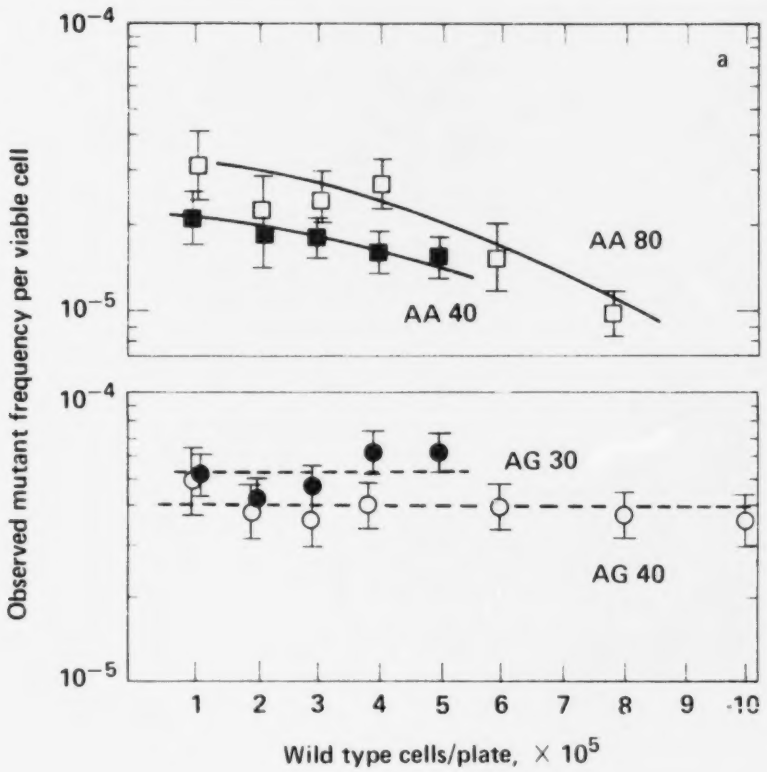
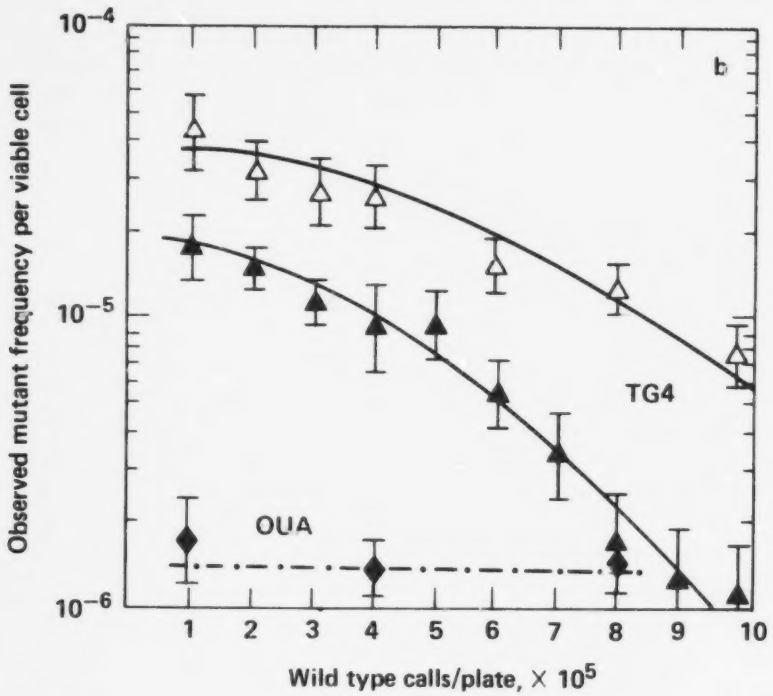



Fig. 4 Reconstruction experiments using two aprt$^{-/-}$, hgprt$^{-}$ sublines derived from CHO-IB-2 cells (one line selected first in AA and then in TG, open symbols, and one line selected in AA followed by AG, closed symbols). The resistant population was plated in 100-mm petri dishes at different IB-2 cell densities (○ = 40 μg/ml AG, □ = 80 μg/ml AA, and △ = 4 μg/ml TG).

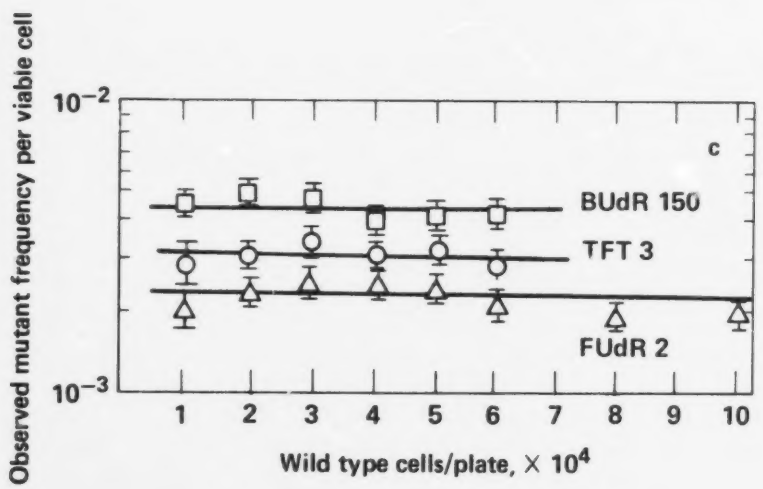



5a

Fig. 5 The observed frequency of spontaneous mutants selected from increasing density of CHO-IB-2 cells (closed symbols) or CHO-AT3-2 cells (open symbols). (a) 30 µg/ml AG (--●--) or 40 µg/ml AG (--○--); 40 µg/ml AA (—■—); or 80 µg/ml AA (—□—). (b) 4 µg/ml TG (—△—, —▲—) or 3 mM OUA (—◆—). (c) 150 µg/ml BUdR (—□—); 3 µg/ml TFT (—○—); 2 µg/ml FUdR (—△—). Error bars are 1 SEM.


b
$10^{-4}$
$10^{-5}$
$10^{-6}$
Observed mutant frequency per viable cell
TG4
OUA
1 2 3 4 5 6 7 8 9 10
Wild type calls/plate, X $10^5$

5b


c
$10^{-2}$
$10^{-3}$
Observed mutant frequency per viable cell
BUdR 150
TFT 3
FUdR 2
1 2 3 4 5 6 7 8 9 10
Wild type cells/plate, X $10^4$

5c

The high spontaneous frequency of $tk^{-/-}$ mutants allows platings below the range where metabolic cooperation is thought to occur. In Fig. 5c the frequency was constant over the range of $1 \times 10^4$ to $1 \times 10^5$ cells/plate ($2 \times 10^2$ to $2 \times 10^3$ cells/cm$^2$); data in Figs. 10a, 10b, and 11d are the result of $3 \times 10^4$ cells/plate in 2 μg/ml FUdR.

Multiple Marker Dose Response for Direct Mutagens. The relative survival ($\bar{S}$), or the fraction of surviving CHO-IB-2 and CHO-AT3-2 cells as a function of applied mutagen dose of MNNG, NQO, or EMS, is given in Fig. 6. Table 6 shows the induction of mutants at three genetic markers ($AA^r$, $AG^r/TG^r$, and $OUA^R$). Detailed dose response data on EMS and MNNG were previously reported for CHO cells (29,30,45); our emphasis was primarily to establish empirically how data for these widely-used, direct-acting mutagens varied as a function of cell line and different culture conditions during expression. Results in Table 6 are pooled from experiments carried out over 1 y using a single lot of FCS; data for experiments using a different lot of serum were consistently elevated and are not shown.

When shown as a function of relative cell survival for all three markers, the response in Table 6 was not always linear at lower cell survival ($< 0.50$). The nonlinearity might reflect unequal growth rates of mutant and wild type cells after higher mutagen doses, resulting in an apparent decline in mutant frequencies. Obtaining mutation data by the replating protocol assumes that both mutant and wild type cells grow at approximately the same rate during expression in suspension culture. We tested this assumption by performing parallel experiments employing either (a) expression of cells in suspension and replating, or (b) *in situ* expression and selection of cells plated at a low cell density (to avoid metabolic cooperation). Results from the *in situ* experiment are also shown in Table 6. Differences in the $AA^r$ mutant frequencies in the replating and *in situ* protocols after 5 to 6 doublings (*in situ*) or 5 to 7 doublings (replating) were significant only for mutants induced by EMS. However, both the $TG^r$ and $AG^r$ mutant frequencies for *in situ* selection were significantly higher for both EMS- and MNNG-induced mutants. Colony-splitting occurring at the time drug medium was added is a possibility, but the correlation of MNNG- and 4-NQO-induced $AA^r$ mutants selected either *in situ* or replated indicates that the magnitude of colony-splitting was variable and small in magnitude, if it occurred at all. Therefore, at least for CHO-IB-2 mutants induced by EMS and MNNG, a subpopulation of $HGPRT^-$ mutants may be at a growth disadvantage in suspension. The growth disadvantage could account for the nonlinearity observed at low survival, where extended growth delays resulting from toxicity obscure the fate of the surviving mutant subpopulation. The delays may negate the assumption that mutant and wild type cells have similar growth properties over the expression period.

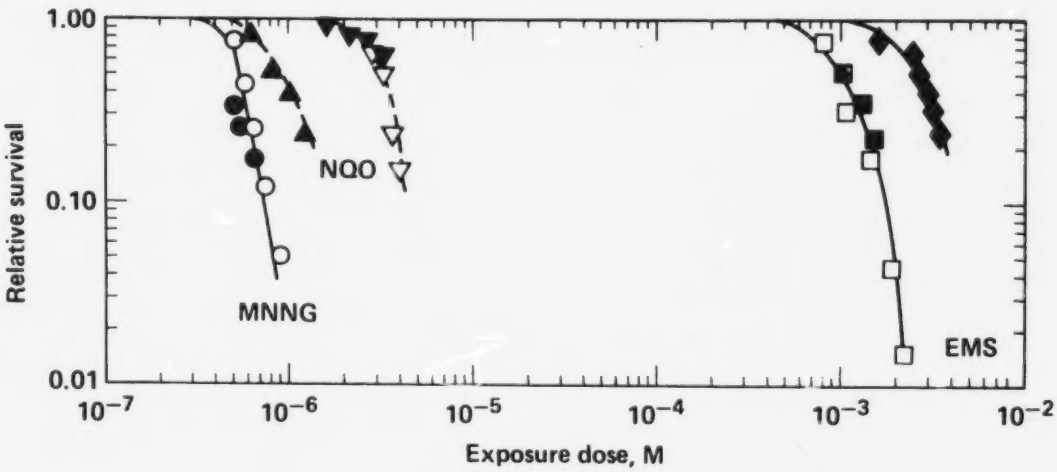



Fig. 6 Decreasing relative survival ($\bar{S}$) of CHO-IB-2 (●,▲,■) or CHO-AT3-2 (▼,♦) cells with exposure dose (M) of direct mutagens MNNG, 4-NQO, and EMS. Solid and open symbols represent separate experiments. CHO-IB-2 cells were treated for 18 h, CHO-AT3-2 cells for 16 h.

Expression may also be carried out in monolayer culture; with expression in monolayer culture, linearity of the dose response has been rereported under conditions of low survival for some mutanges (29,30) but not others (61,62). The multiple marker mutagenesis assay reported here requires a large number of cells per dose point, e.g. about $5 \times 10^7$ for spontaneous and low dose determinations, if optimal statistical parameters are desired. The need to maintain cell density below a critical level in monolayer culture during a prolonged expression period (1,13) makes monolayer expression impractical for these large-scale experiments. Transfer of the cells to suspension culture for two or more doublings before drug selection appears to be sufficient to minimize cell-to-cell interaction before selection (Carver, unpublished data).

Metabolic Activation. At a dose of 9 mM DMN, maximal induction of mutation for all four markers was obtained with concentrations ≤ 1.6 mg/ml S-9 protein; a similar increase in DMN-induced mutants as a function of cell survival was observed when microsomal activation ( ≤ 0.75 mg/ml) was used. When S-9 protein concentrations were ≥ 2.0 mg/ml S-9 or ≥ 1.0 mg/ml microsomes, toxicity was not increased (Fig. 7) and the induced mutant frequency (data not shown) remained level or decreased.

TABLE 6 Induced mutant frequency of CHO-IB-2 cells treated with direct mutagens[a]

| | μg/ml | S | $AA^r \times 10^{-4}$ | $AG^r \times 10^{-4}$ | $TG^r \times 10^{-4}$ | $OUA^R \times 10^{-5}$ |
|---|---|---|---|---|---|---|
| EMS[b] | 100 | 0.74 | 2.4 ± 0.2 | 4.7 ± 0.2 | 1.1 ± 0.1 | 2.5 ± 0.2 |
| | 125 | 0.30 | 7.3 ± 0.3 | 14.1 ± 0.8 | 5.0 ± 2.5 | 10.0 ± 0.5 |
| | 175 | 0.17 | 9.7 ± 0.5 | 20.5 ± 1.2 | 7.0 ± 0.4 | 9.6 ± 0.5 |
| | 225 | 0.04 | 7.4 ± 0.4 | - | 7.7 ± 0.3 | 8.9 ± 0.5 |
| | 275 | 0.02 | 11.3 ± 0.6 | - | 8.4 ± 0.4 | 13.1 ± 0.6 |
| (In Situ) | 125 | 0.30 | 20.0 ± 3.8 | 108.8 ± 16.3 | 34.8 ± 2.6 | - |
| NQO[b] | 0.30 | 0.96 | 0.1 ± 0.01 | 0.2 ± 0.0 | 0.4 ± 0.31 | 0.5 ± 0.04 |
| | 0.40 | 0.83 | 0.6 ± 0.04 | 1.5 ± 0.1 | 0.7 ± 0.5 | 1.5 ± 0.1 |
| | 0.50 | 0.77 | 2.2 ± 0.2 | 2.6 ± 0.2 | 1.7 ± 0.2 | - |
| | 0.55 | 0.66 | 3.5 ± 0.4 | 10.1 ± 0.8 | 3.1 ± 0.3 | 3.0 ± 0.3 |
| | 0.65 | 0.49 | 5.4 ± 0.4 | 16.8 ± 1.1 | 4.9 ± 0.5 | 3.9 ± 0.4 |
| | 0.70 | 0.15 | 6.2 ± 0.4 | 19.0 ± 2.0 | 6.3 ± 0.4 | 5.4 ± 0.5 |
| (In Situ) | 0.65 | 0.49 | 3.0 ± 1.2 | 18.8 ± 5.6 | 9.3 ± 1.2 | - |
| (In Situ) | 0.70 | 0.15 | 5.6 ± 2.2 | 35.8 ± 10.7 | 10.4 ± 2.1 | - |
| MNNG[b] | 0.07 | 0.75 | 1.1 ± 0.1 | 0.8 ± 0.1 | - | 0.3 ± 0.05 |
| | 0.08 | 0.44 | 2.2 ± 0.1 | 6.7 ± 0.4 | 0.6 ± 0.1 | - |
| | 0.09 | 0.26 | 2.2 ± 0.2 | 7.1 ± 0.5 | 0.8 ± 0.1 | 1.6 ± 0.1 |
| | 0.11 | 0.12 | 2.7 ± 0.2 | 7.1 ± 0.5 | 1.0 ± 0.1 | 2.1 ± 0.2 |
| | 0.14 | 0.05 | 2.8 ± 0.2 | 7.7 ± 0.7 | 1.1 ± 0.1 | 2.4 ± 0.2 |
| (In Situ) | 0.11 | 0.12 | 5.9 ± 2.4 | 49.5 ± 14.9 | 6.8 ± 2.6 | - |

[a]Spontaneous frequencies were as follows: $AA^r$ $1.2 \times 10^{-4}$; $AG^r$ $1.0 \times 10^{-4}$; $TG^r$ $4.6 \times 10^{-5}$; $OUA^R$ $5.1 \times 10^{-6}$.

[b]Treatment time was 18 h.

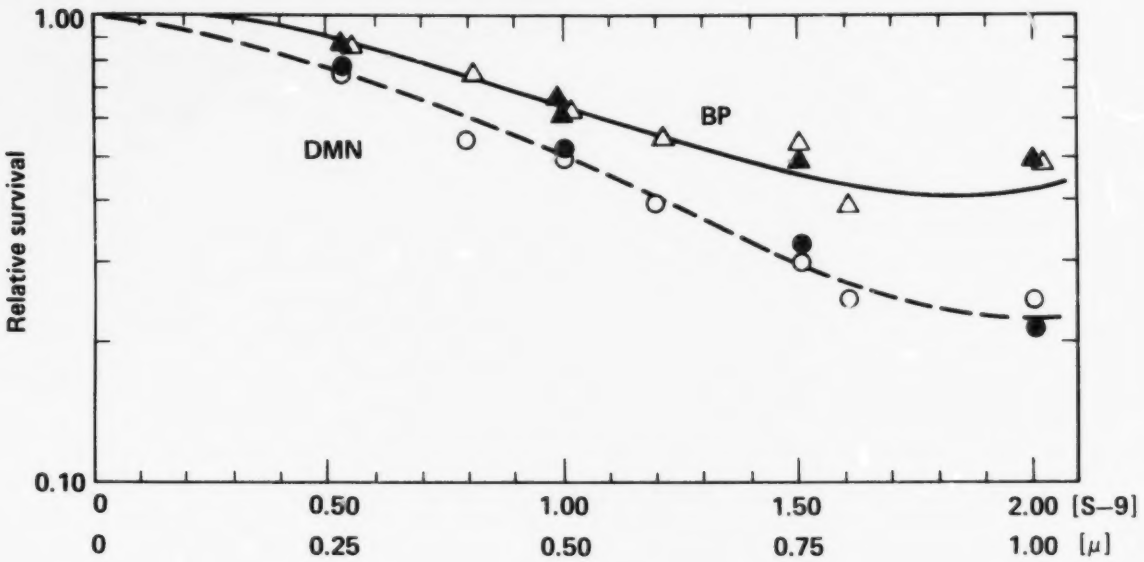



Fig. 7 Decreasing relative survival ($\bar{S}$) of CHO-AT3-2 cells with increasing S-9 (open symbols) or microsome (μ, solid symbols) concentrations (mg/ml total protein). The cells were treated for 2 h with the promutagens DMN at 9 mM (○,●) and BP at 0.04 mM (△,▲).

When activation of BP by S-9 at concentrations of 0.50 to 2.0 mg/ml was compared to activation by microsomes at 0.25 to 1.0 mg/ml, the data indicated that BP was most effectively metabolized to mutagenic products by microsomes at ≤ 0.50 mg/ml; microsome concentrations > 0.50 mg/ml consistently lowered the optimal yield of BP-induced mutants. Although we observed only minimal induced mutation from BP activated by S-9 at > 0.5 mg/ml, recent experiments have indicated that BP is efficiently converted to mutagenic metabolites by S-9 at ≤ 0.1 mg/ml, followed by a decrease at higher concentrations of S-9 protein.

Determination of Expression Time. Following induction of (genotypic) mutation, a period, i.e., expression time, is required to allow maximal phenotypic expression before drug selection. In these experiments, the mutations were expressed during cell growth in suspension culture to minimize cell-to-cell contact and to provide uniform growth conditions. The CHO-AT3-2 subline had a doubling time of 14 h in suspension culture, and the spontaneous frequency of all four drug-resistant loci remained reasonably constant (S.D. of ± 60%) over a 10 d period. The relative survival for CHO-AT3-2 exposed to EMS is shown in Fig. 6. Fig. 7 shows the relative survival ($\bar{S}$) of CHO-AT3-2 cells as a function of increasing

S-9 or microsome concentration; promutagens coupled with metabolic activion were DMN (9 mM) and BP (0.04 mM).

In the multiple-marker system, expression of $AA^r$ mutants induced by EMS was complete by 2 d (Fig. 8a). The BP-induced $AA^r$ mutant frequency (Fig. 8b) appeared to be maximal by 4 d, declining slightly but not significantly over the next 6 d. The DMN-induced $AA^r$ mutant phenotypes (Fig. 8c) were maximally expressed by 3 d, and a plateau in induced mutant frequency was maintained through 10 d. The longer expression time needed for complete expression of TG resistance (compared to AG resistance) has been documented previously (28). Mutants induced by EMS showed full TG-resistance by 6 d (Fig. 9a). The frequency of $TG^r$ mutants in response to BP was also maximal by 6 d, with no increase observed during the following 4 d (Fig. 9b). The DMN-induced mutant frequency for $TG^r$ shown in Fig. 9c increased up to 8 d and had plateaued by 10 d.

In Fig. 10a, $OUA^R$ mutants were fully expressed by 3 d and remained reasonably constant over the next 7 d. With CHO-IB-2 cells, we observed a pronounced decline in the number of MNNG-induced $OUA^R$ mutants after 10-12 d (data not shown). The expression of $FUdR^r$ mutants is shown in Figs. 10b and 10c. Mutants induced by EMS were maximal at 2-3 d, then declined. However, the response to BP declined after 1 d. The frequency of mutants induced by DMN was maximal at 3 d, declining significantly at 4 d and then remaining constant over the 10 d period. Response at the tk locus following exposure to several mutagens has shown maximal expression at 1-4 d (Adair and Carver, in preparation).

Multiple Marker Dose Response for Optimal Expression. In Figs. 11a-11d, the induced mutant frequency per viable cell is fitted as a linear function of the response to mutagen dose in units of the logarithym of decreasing cell survival. The slope of the linear function can be used to compare the mutagenic potency at equivalent cell survival. If the $AA^r$ response (Fig. 11a) after BP treatment is compared to that for DMN, i.e. a ratio of the slopes, then DMN and BP are comparable mutagens at the aprt locus ($P \geq 0.80$). In terms of equal cell survival, EMS is about seven times more potent than DMN or BP. In Fig. 11b, the response of $TG^r$ mutants induced by BP is shown. The response is slightly less than that for DMN ($P \leq 0.06$ that the mutagens are comparable in potency). Similar to $AA^r$, the $TG^r$ response for EMS is about six fold greater than that for DMN or BP. In Fig. 11c, BP and DMN are similar in potency ($P \sim 0.30$) for inducing $OUA^R$ mutants. The response for EMS at this locus is about seven times greater than that of DMN and BP.

The above data have compared the mutant frequency observed at optimal (7-8 d) expression. For the tk locus, the data are compared in Fig. 11d for optimal but varying expression, i.e. 1 d for BP, 3 d for DMN and EMS. At early expression, DMN and BP are comparable mutagens; however, EMS is slightly less effective than DMN and BP at the tk locus.

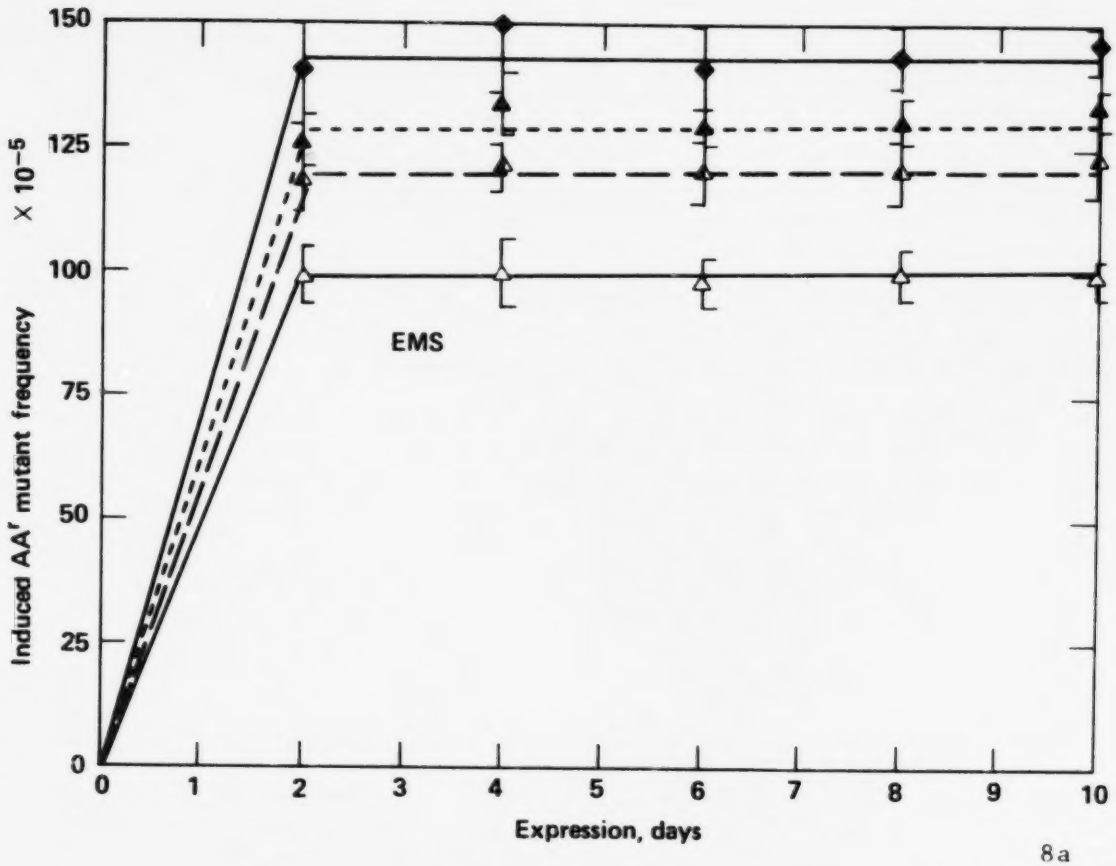
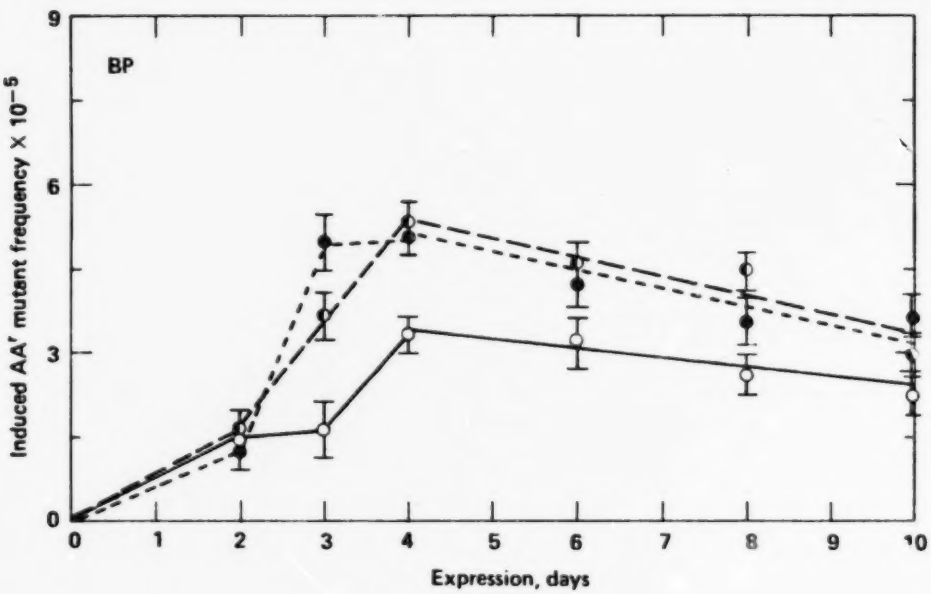



Fig. 8 Induced $AA^r$ mutant frequency per viable cell (CHO-AT3-2) as a function of expression time before selection with 80 μg/ml AA. Error bars are 1 SEM. The $AA^r$ spontaneous frequency (± 1 S.D.) was $1.35 \pm 0.85 \times 10^{-5}$. The mutagen concentrations and corresponding fraction of surviving cells at time zero were as follows:

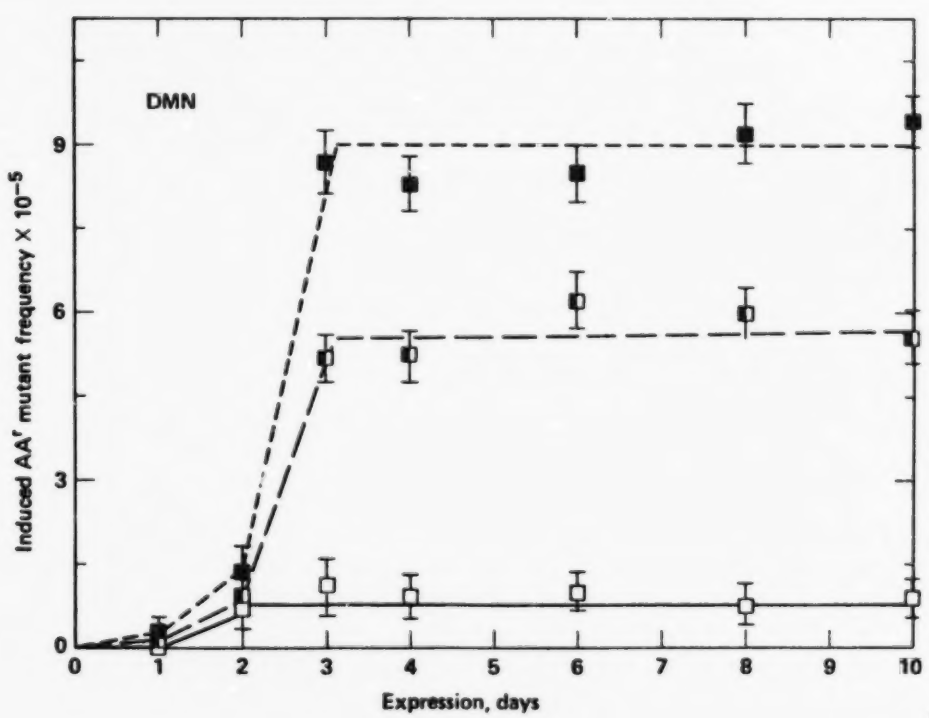
(a) EMS: 2.4 mM, 0.53 (—△—); 2.9 mM, 0.40 (– –▲– –);
3.2 mM, 0.33 ( --▲-- ); 3.4 mM 0.24 (—♦—).

(b) BP: 0.04 mM, 0.2 mg/ml S-9, 0.90 (—○—);
0.04 mM, 0.4 mg/ml S-9, 0.76 (– –●– –);
0.04 mM, 0.6 mg/ml S-9, 0.69 ( - -●- - ).

(c) DMN: 9 mM, 0.5 mg/ml S-9, 0.94 (—□—);
9 mM, 1.0 mg/ml S-9, 0.80 (– –■– –);
9 mM, 1.5 mg/ml S-9, 0.55 ( - -■- - ).


BP
Induced AA' mutant frequency X 10⁻⁵
0
3
6
9
0 1 2 3 4 5 6 7 8 9 10
Expression, days

8b


DMN
Induced AA' mutant frequency X 10⁻⁵
0
3
6
9
0 1 2 3 4 5 6 7 8 9 10
Expression, days

8c

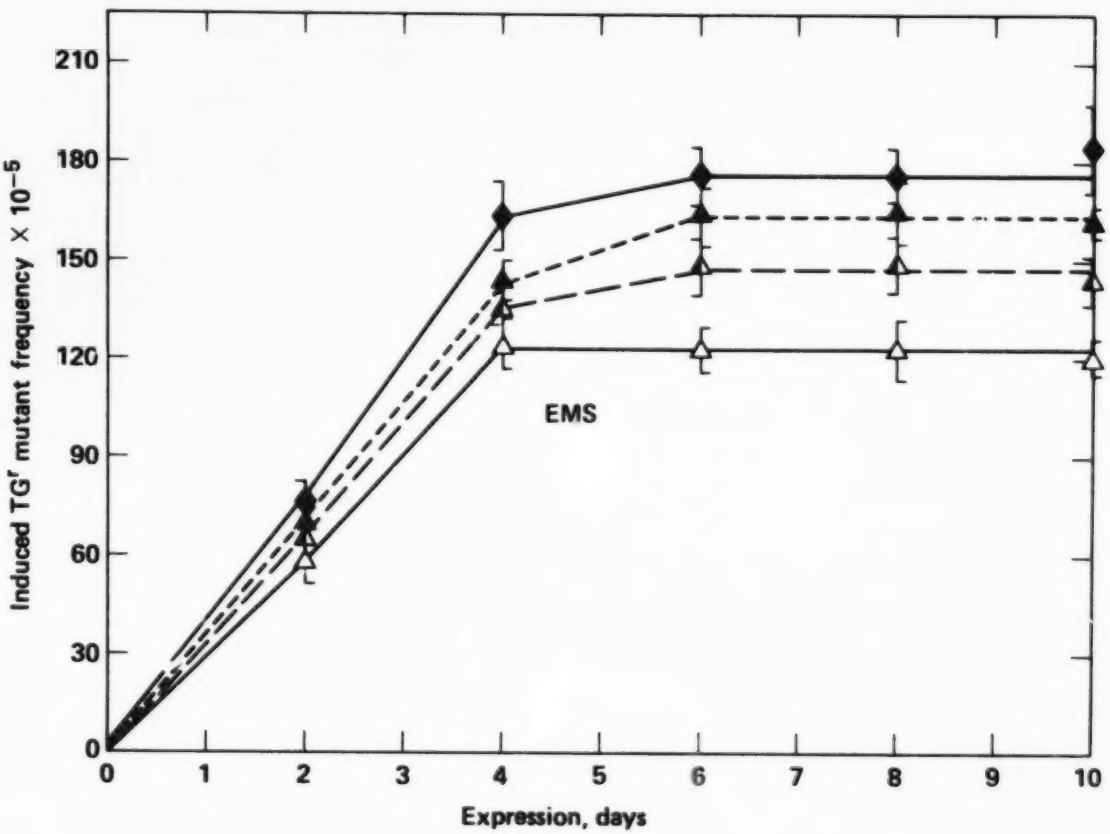
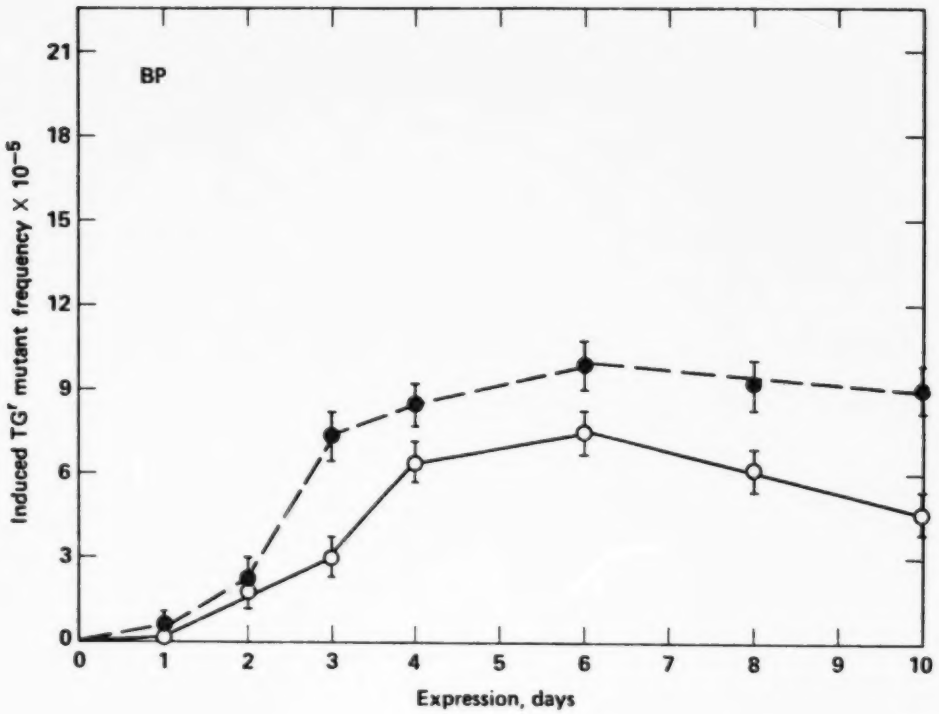
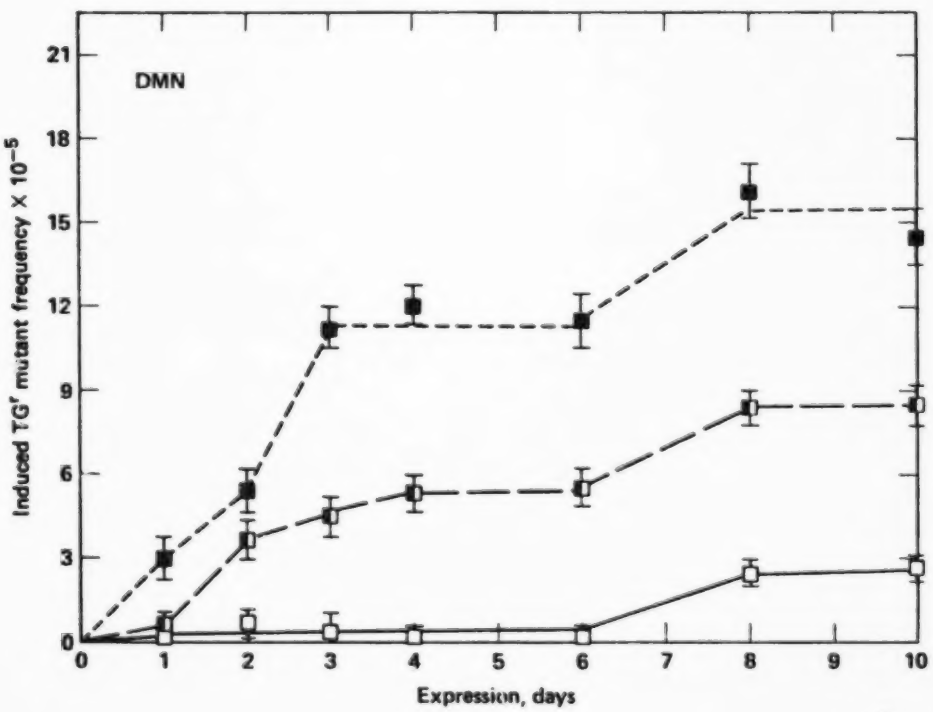


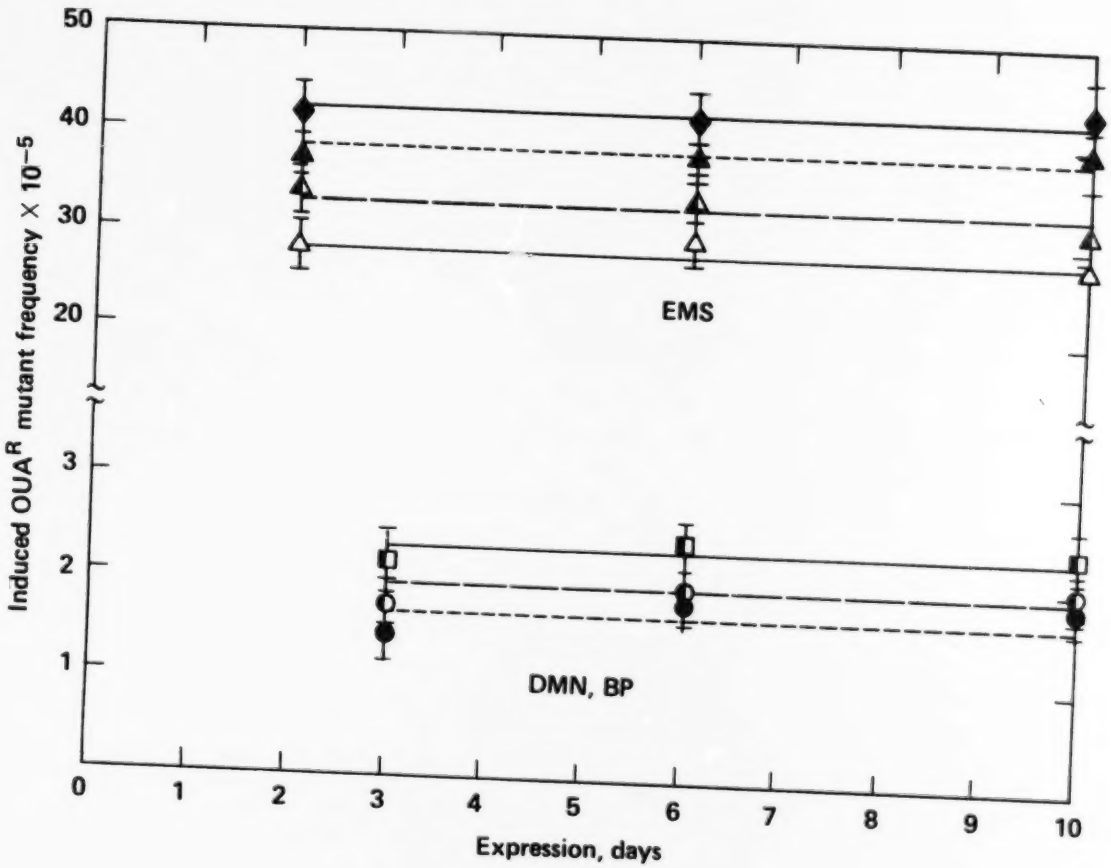

Fig. 9 Induced $TG^r$ mutant frequency per viable cell (CHO-AT3-2) as a function of expression time before selection with 4 µg/ml AA. All symbols as in Fig. 8. Spontaneous $TG^r$ frequency was $2.52 \pm 1.46 \times 10^{-5}$.


BP
Induced TG$^r$ mutant frequency X $10^{-5}$
0
3
6
9
12
15
18
21
0 1 2 3 4 5 6 7 8 9 10
Expression, days

9b

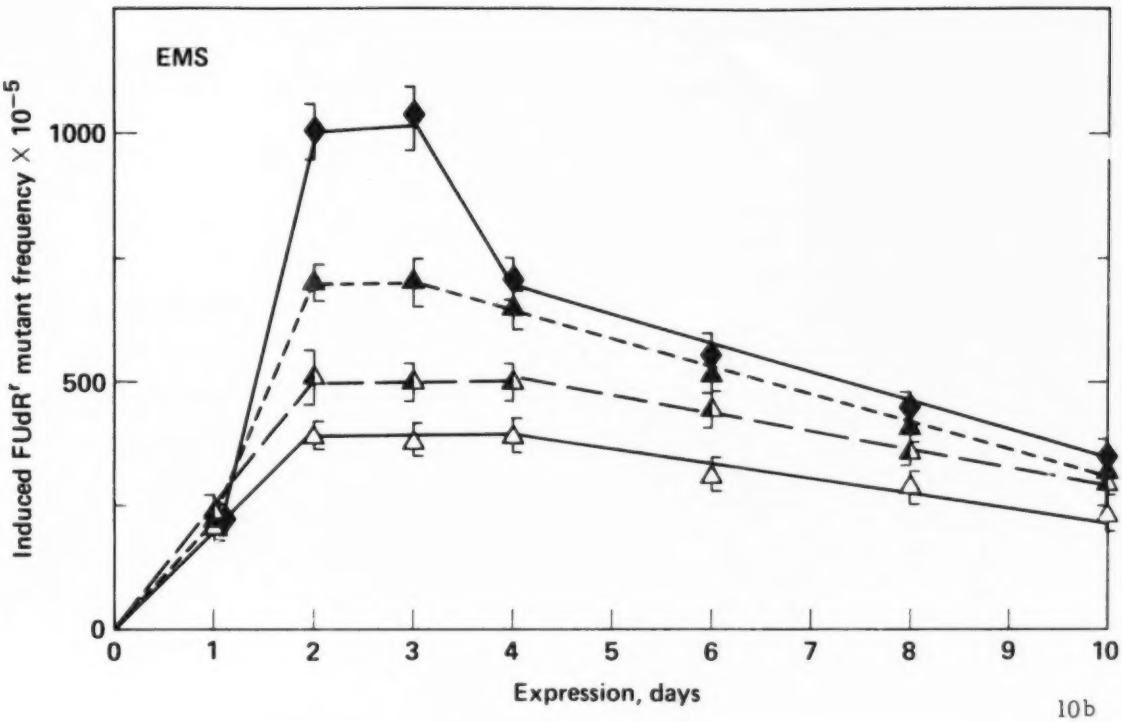

DMN
Induced TG$^r$ mutant frequency X $10^{-5}$
0
3
6
9
12
15
18
21
0 1 2 3 4 5 6 7 8 9 10
Expression, days

9c

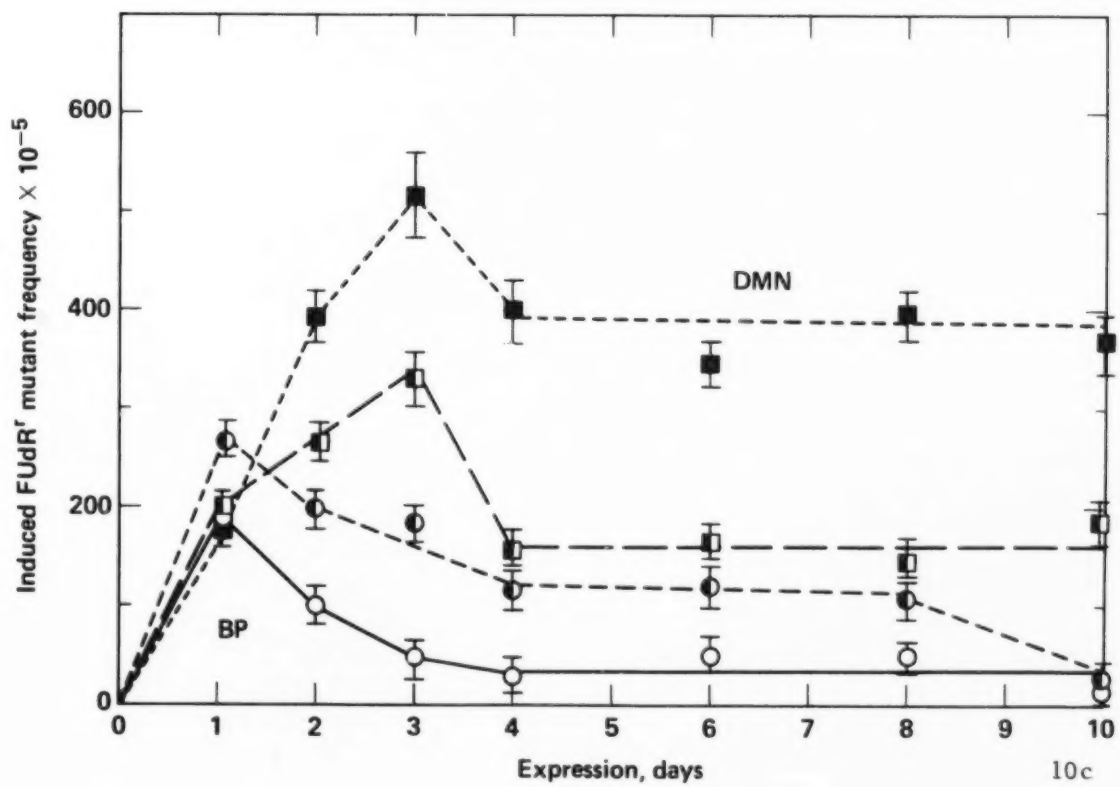



10a

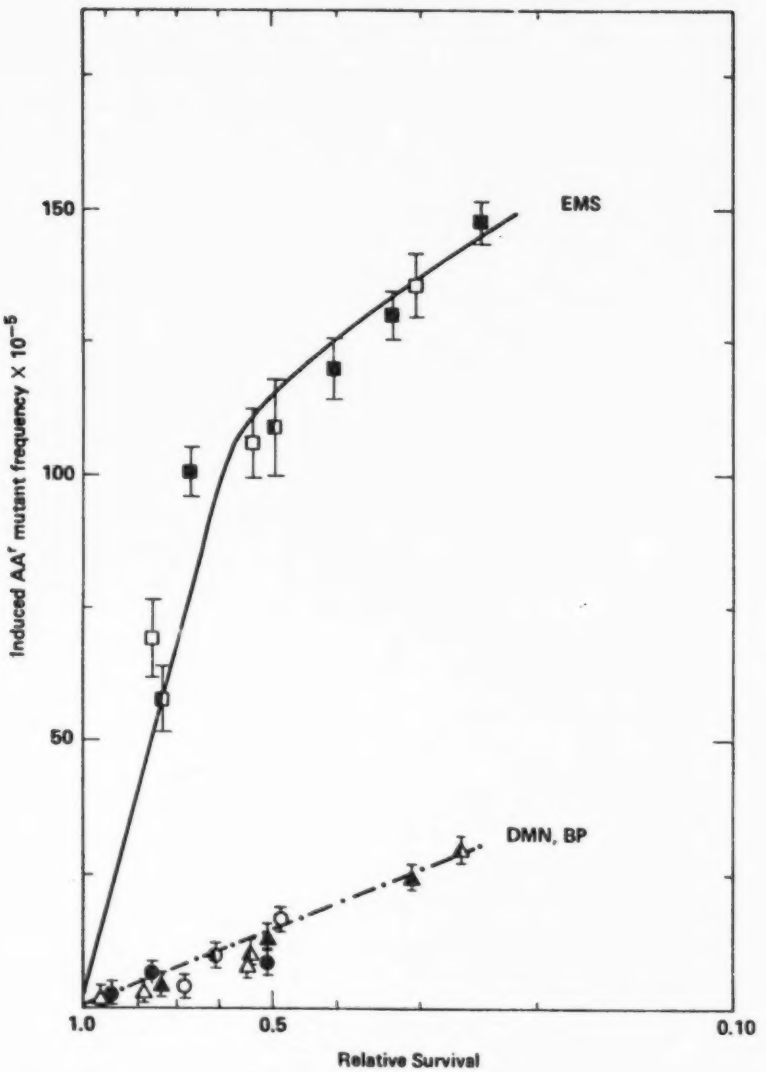
Fig. 10 Induced FUdR$^r$ or OUA$^R$ mutant frequency per viable cell (CHO-AT3-2) as a function of expression time before selection with 2 μg/ml FUdR or 3 mM OUA. All symbols as in Fig. 8. Spontaneous frequency was $2.44 \pm 0.81 \times 10^{-6}$ for OUA$^R$ and $2.39 \pm 0.86 \times 10^{-3}$ for FUdR$^r$.


EMS
Induced FUdR$^r$ mutant frequency $\times 10^{-5}$
1000
500
0
0 1 2 3 4 5 6 7 8 9 10
Expression, days

10b


Induced FUdR$^r$ mutant frequency $\times 10^{-5}$
600
400
200
0
DMN
BP
0 1 2 3 4 5 6 7 8 9 10
Expression, days

10c




11a

Fig. 11a Increase in induced frequency of $AA^r$ mutants (7-8 d expression) with decreasing relative survival or fraction of surviving CHO-AT3-2 cells (S) after treatment with DMN (-·-▲-·-), BP (-·-●-·-), or EMS (—■—). Open and closed symbols represent different experiments. The linear regression of the straight line fit ($y = b_0 + b_1 X$, where $X = \log S$ ) has the following parameters: ($\pm$ 1 S.D.):

DMN: $b_0 = -0.27 \pm 0.86 \times 10^{-5}$, $b_1 = 4.83 \pm 0.28 \times 10^{-4}$;

BP: $b_0 = -1.75 \pm 1.57 \times 10^{-5}$, $b_1 = 5.04 \pm 0.92 \times 10^{-4}$;

EMS: $b_0 = 1.72 \pm 1.62 \times 10^{-4}$, $b_1 = 3.33 \pm 0.77 \times 10^{-3}$.

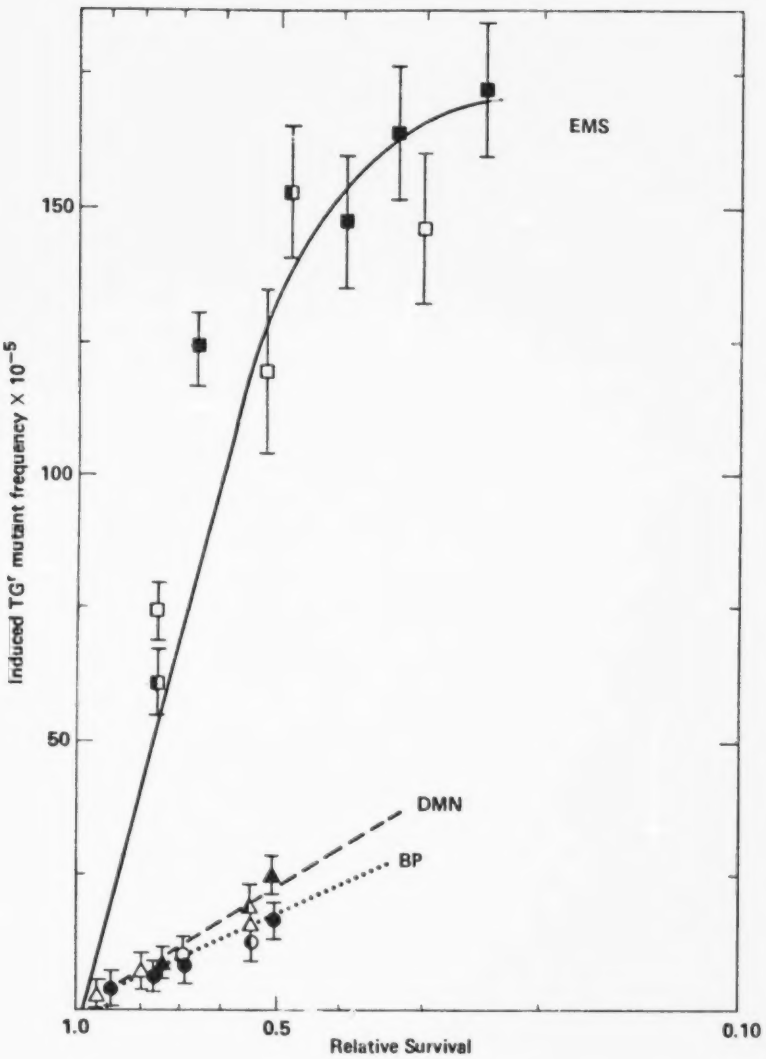



Fig. 11b Increase in induced frequency of TG$^r$ mutants (7-8 d expression) with decreasing relative survival or fraction of surviving CHO-AT3-2 cells ($\bar{S}$) after treatment with DMN, BP, or EMS. All symbols as in Fig. 11a. Regression parameters are:

DMN: $b_0 = 0.26 \pm 0.92 \times 10^{-5}$, $b_1 = 7.66 \pm 0.35 \times 10^{-4}$;

BP: $b_0 = -1.69 \pm 1.58 \times 10^{-5}$, $b_1 = 5.45 \pm 0.85 \times 10^{-4}$;

EMS: $b_0 = 1.55 \pm 1.95 \times 10^{-4}$, $b_1 = 4.43 \pm 0.93 \times 10^{-3}$.

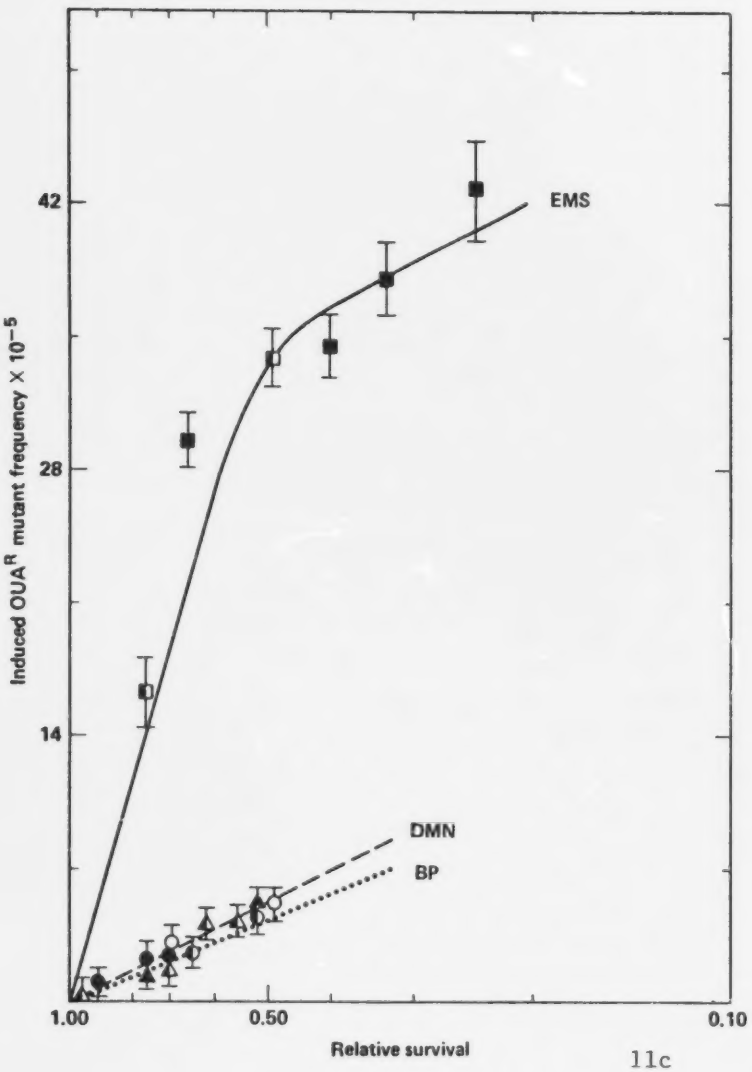



Fig. 11c Increase in induced frequency of OUA$^R$ mutants (7-8 d expression) with decreasing relative survival or fraction of surviving CHO-AT3-2 cells ($\bar{S}$) after treatment with DMN, BP, or EMS. All symbols as in Fig. 11a. Regression parameters are:

DMN: $b_0 = 0.22 \pm 2.1 \times 10^{-6}$, $b_1 = 1.63 \pm 0.12 \times 10^{-4}$;

BP: $b_0 = 0.12 \pm 3.22 \times 10^{-6}$, $b_1 = 1.42 \pm 0.17 \times 10^{-4}$;

EMS: $b_0 = 3.00 \pm 4.69 \times 10^{-5}$, $b_1 = 1.11 \pm 0.25 \times 10^{-3}$.

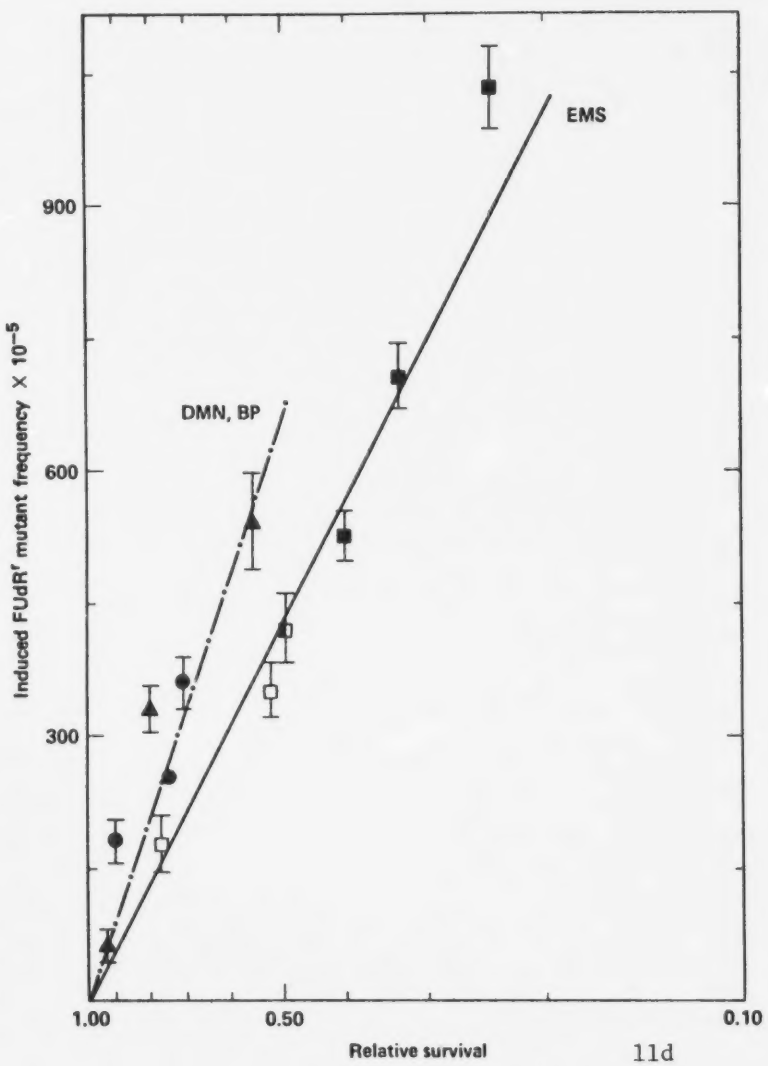



Fig. 11d Increase in induced frequency of FUdR$^r$ mutants after optimal expression time with decreasing relative survival or fraction of surviving CHO-AT3-2 cells ($\bar{S}$) after treatment with DMN, BP, or EMS. All symbols as in Fig. 11a. Regression parameters are:

DMN (3 d): $b_0 = 0.47 \pm 0.34 \times 10^{-3}$, $b_1 = 1.93 \pm 0.26 \times 10^{-2}$;

BP (1 d): $b_0 = 0.47 \pm 0.34 \times 10^{-3}$, $b_1 = 1.93 \pm 0.26 \times 10^{-2}$;

EMS (3 d): $b_0 = 0.14 \pm 0.07 \times 10^{-3}$, $b_1 = 1.38 \pm 0.20 \times 10^{-2}$.

Validation of Mutant Phenotypes. As shown in Fig. 12a, of the 61 $AA^r$ clonal isolates assayed for APRT activity, 96% (22/23) of the spontaneous mutants and 82% (31/38) of the mutants from mutagenized cultures had ≤ 5% wild type activity; only 4 $AA^r$ clones had APRT activity approaching those of the heterozygous parental cells. Of the 54 $AG^r$ clones assayed for HGPRT activity, 89% (16/18) of the spontaneous mutants and 86% (31/36) of the mutants from mutagenized cultures had ≤ 30% wild type activity; 4 $AG^r$ clones had HGPRT activities similar to those of CHO-IB-2 and CHO-AT3-2. The mean residual HGPRT activity of $TG^r$ mutants was much lower than that of $AG^r$ mutants. None of the 30 $TG^r$ mutant clones assayed had WT HGPRT activity, with 94% (17/18) of the spontaneous mutants and 67% (8/12) of the induced mutants having ≤ 5% WT activity.

In Fig. 12b, the residual TK activity of spontaneous and induced mutants is shown. Because the spontaneous frequency of $FUdR^r$ is high, no attempt was made to distinguish between spontaneous and induced mutants. Both classes had virtually no detectable TK activity; a single isolated mutant appeared to have 15% of WT TK activity, but the line was lost before the assay could be repeated. All other mutants have had ≤ 1% WT activity. After treatment with DMN or BP, fifty induced mutant clones resistant to 2 μg/ml FUdR were isolated. The clones were grown 12-16 generations in the absence of FUdR, then tested for $FUdR^r$, $BUdR^r$, $TFT^r$, and $HAT^s$. All fifty clones displayed cross-resistance to the three thymidine analogs (P.E. 0.40-0.70) and showed complete sensitivity to HAT (P.E. < $10^{-4}$).

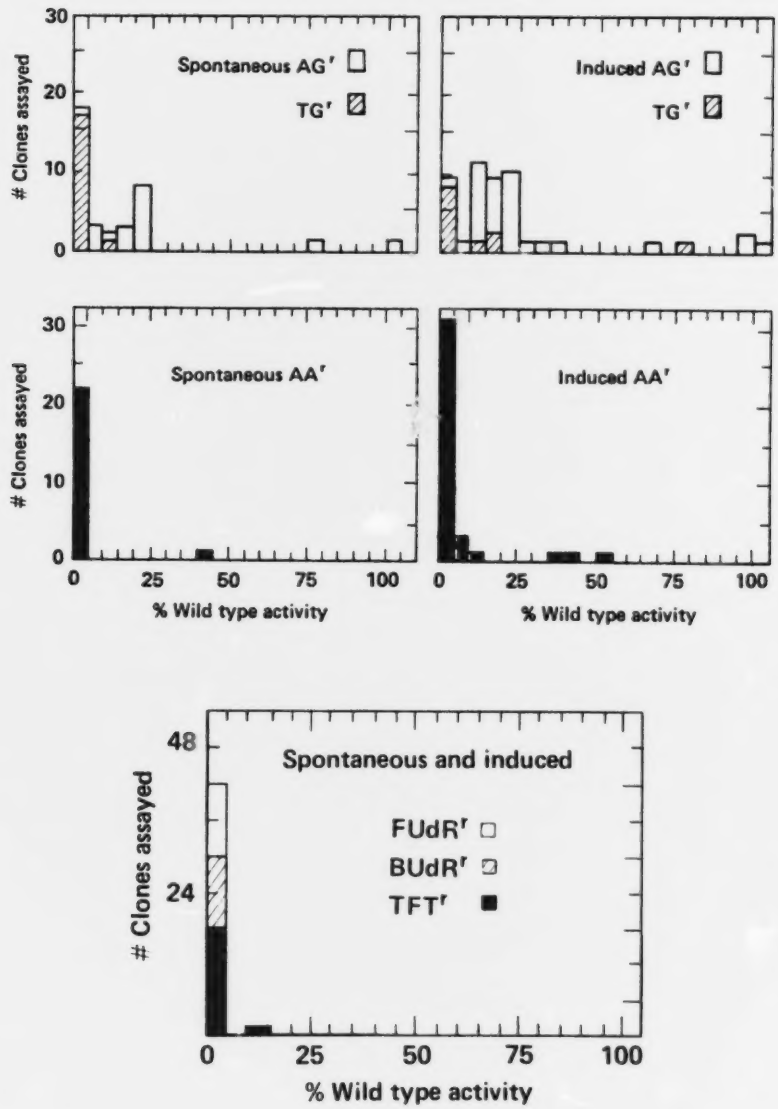



Fig. 12 Activities of HGPRT, APRT, or TK (relative to wild type) for spontaneous and induced mutants. Mutant clones were isolated, grown 10-12 generations in the absence of selecting drug, and assayed for in vitro activity of the appropriate enzyme. (a) Mutants resistant to 40 µg/ml AG or 4 µg/ml TG or 80 µg/ml AA; (b) mutants resistant to 2 µg/ml FUdR, 150 µg/ml BUdR, and 3 µg/ml TFT.

## SECTION 6
## DISCUSSION

As an *in vitro* test system, CHO cells combine a near diploid karyotype that facilitates genetic and cytogenetic analysis with the favorable growth characteristics, e.g., high plating efficiency and rapid growth in either monolayer or suspension culture, necessary for large-scale, cost-effective mutagen screening. The CHO-AT3-2 subline, which is also heterozygous at both the *aprt* and *tk* loci, was developed to allow single-step selection of autosomal recessive $AA^r$ and $FUdR^r$ mutant phenotypes, as well as the more commonly used X-linked $TG^r$ and co-dominant $OUA^R$ genetic markers. Although functionally heterozygous *aprt*$^{+/-}$ strains of CHO had been isolated previously (17,52), optimal conditions for the phenotypic expression and selection of $AA^r$ mutants were not established, and the strains were not extensively used for quantitative mutagenesis assays. Only limited data have been reported in the literature regarding the optimizations of selection parameters and the induction of APRT-deficient mutants by known mutagens (17,34,52,87,92). Presumptive *tk*$^{+/-}$ revertants of TK-deficient mutants have been isolated in several cell lines (3,10,22,80,85). Extensive validation and application of the L5178Y *tk*$^{-/-}$ mouse lymphoma system for mutagenesis assays (20,22,24,25) prompted our efforts to use the *tk* locus in a CHO cell mutagenesis assay system. The CHO-AT3-2 subline facilitates simultaneous, quantitative mutagenicity testing at four specific gene loci.

*Derivation of heterozygous cell lines.* Phenotypic expression of single-step forward mutations at the autosomal *aprt* and *tk* loci, and use of the $AA^r$ and $FUdR^r$ genetic markers for mutagenesis assay, first required the derivation of a CHO subline that was heterozygous for these loci. Using the approach of Jones and Sargent (52), an *aprt* $^{+/-}$ heterozygote with approximately 50% wild-type APRT activity (CHO-IB-2) was selected from an unmutagenized CHO cell population. The *aprt* $^{+/-}$ CHO-IB-2 cells were subsequently used to derive sublines that were also heterozygous for the *tk* locus. Since direct, single-step selection of *tk* $^{+/-}$ heterozygotes proved to be impractical (2,22,67), such heterozygotes were obtained by first isolating rare, $BUdR^r$, *tk* $^{-/-}$ mutants of CHO-IB-2, and subsequently selecting $HAT^r$ revertants which had intermediate levels of TK activity. A similar approach was used by Clive and co-workers (22) to obtain the *tk* $^{+/-}$ heterozygote employed in their L5178Y TK $^{+/-}$ mouse lymphoma mutagenesis assay system.

*Biochemical and genetic characterization.* The CHO-AT3-2 cells are heterozygous for both the *aprt* and *tk* loci. Consistent with this premise

were: (a) the manner in which the CHO-AT3-2 cell line was derived (Fig. 1 & 2); (b) this line's intermediate levels of APRT and TK activity (Fig. 1 & 2); (c) the high frequencies at which APRT- or TK-deficient mutants were obtained from unmutagenized CHO-AT3-2 cell populations in single-step selections with AA or FUdR (Table 1, Fig. 3); and (d) forward mutation rates at these autosomal gene loci that were greater than or similar to those at the X-linked hgprt locus (Tables 2 & 3). Spontaneous and EMS-induced frequencies of $AA^r$ and $BUdR^r$ or $FUdR^r$ mutants were at least 2-3 orders of magnitude higher in CHO-AT3-2 cells than in wild-type, aprt $^{+/+}$ and tk $^{+/+}$ CHO cells (Table 1). Segregation analysis (Table 5) confirmed that CHO-AT3-2 cells were indeed functionally hemizygous for the genes for APRT and TK.

Biochemical validation of drug resistant mutant phenotypes revealed that fifty-one of fifty-nine clones selected on the basis of their resistance to AA had ≤ 5% of wild-type APRT activity. APRT-deficient mutants have been selected from several other cells lines (17,33,52,87,-92,93), and the aprt locus has been mapped in both the mouse (59) and human (93) karyotypes. Functionally heterozygous, aprt $^{+/-}$ strains have not been previously utilized for quantitative mutagenesis assay, and only limited data has been reported in the literature regarding the induction of AA-resistance by known mutagens (15,16,52,92). Dose-dependent increases in the frequency of $AA^r$ colonies (~10 fold over background) after treatment of an aprt $^{+/-}$ strain of CHO cells with EMS were reported by Jones and Sargent (52), but expression kinetics for the $AA^r$ marker were not determined. Having established optimal conditions for the phenotypic expression and selection of $AA^r$ mutants in our system (15.16), we can directly compare induced mutation frequencies at the aprt and hgprt loci, and assess the relative sensitivity of the genes for APRT and HGPRT to mutation by various physical and chemical agents. For the direct-acting mutagen, EMS, induced mutation frequencies for AA-resistance were only slightly lower than those for TG-resistance (Fig. 3). However, we have observed mutagen-specific differences in the relative mutability of the aprt and hgprt loci using other mutagens (15,16; Adair & Carver, manuscript in preparation).

Although TK-deficient strains have been isolated from a number of different cell lines (10,22,55-57,80,85,86), many of these variants were obtained by prolonged, multi-step selection with BUdR, and are of uncertain genetic origin. The presumptive structural gene locus for TK has been mapped in both the mouse (60) and human (36,66) karyotypes, using TK-deficient mutants of mouse and Chinese hamster cells. Derivation of a tk $^{+/-}$ heterozygote of L5178Y mouse lymphoma cells by Clive and co-workers (22), and extensive validation and application of this system for mutagenesis assay (21-23,25,26,67), has prompted efforts to utilize the tk locus in other mammalian cell mutagenesis assay systems as well (16,85).

We have isolated TK-deficient mutants of CHO cells using BUdR, FUdR, or TFT as the selecting agent. All but one of forty-three thymidine analog-resistant clones isolated from CHO-AT3-2 or CHO-IB-2 had ≤ 1% of wild-type TK activity. All of the TK-deficient mutants we have tested were HAT-sensitive and cross-resistant to BUdR, FUdR, and TFT. Further

biochemical and genetic characterization of several TK-deficient mutants and $HAT^r$ revertants with intermediate levels of TK activity provided evidence that they probably represent mutations in the structural gene for TK (Adair, manuscript in preparation). The thymidine analog-resistant, TK-deficient mutant phenotypes were recessive, and complementation analysis (Table 4) indicated that all of the $BUdR^r$, $FUdR^r$, or $TFT^r$ clones tested represented the same complementation group, reflecting mutation at the same genetic locus. Furthermore, non-complementation of the thymidine analog-resistant phenotype in hybrids with TK-deficient DON-a3 cells identified this genetic locus as the presumptive structural gene locus for TK, previously mapped in both the mouse and human genomes using the DON-a3 strain (36,60).

Most previous studies have utilized BUdR as the selecting agent for TK-deficient mutants (10,21-23,25,26,40,55-57,80). The killing of TK-positive wild-type cells by this drug is rather slow, and several cell divisions can occur before cell death (2,42,67,94). FUdR rapidly inhibits cell division (42,94), effectively eliminating background growth of wild-type cells; unlike BUdR, it is apparently not mutagenic in mammalian cells (50). Both analogs are readily phosphorylated by TK, but FUdR is not further metabolized to the di- or tri-phosphates, or incorporated into DNA (42,43). FUdMP is a potent, irreversible inhibitor of thymidylate synthetase (43,79). The observed cross-resistance of TK-deficient mutants to BUdR, FUdR, and TFT, along with the non-complementation of TK-deficient mutants regardless of which drug was used for their selection (Table 4) and the similar rates of spontaneous mutation for resistance to each drug in CHO-AT3-2 (Table 3), together suggest that BUdR and FUdR select for the same class of TK-deficient mutant phenotypes. Therefore, we have used FUdR as the selecting agent for such mutants in these CHO-AT3-2 multiple-marker mutagenesis experiments.

The tk locus appears to be considerably more susceptible to spontaneous mutation than the genes for APRT, HGPRT, or $Na^+/K^+$ ATPase ($OUA^R$) in Chinese hamster cells. Background frequencies of $BUdR^r$ or $FUdR^r$ colonies for unmutagenized CHO-AT3-2 cell populations were consistently $1.5 - 3.0 \times 10^{-3}$. Similar spontaneous frequencies have been reported for $BUdR^r$ variants in several presumptive $tk^{+/-}$ heterozygotes of another Chinese hamster cell line (80). Luria-Delbruck fluctuation analysis (Table 3) indicated that the high frequencies of thymidine analog-resistant variants in CHO-AT3-2 cell populations reflect a very high spontaneous rate of mutation at the tk locus. Stable, thymidine analog-resistant variants arise spontaneously at a rate of $2\text{-}3 \times 10^{-4}$ per cell per generation; this rate is approximately three orders of magnitude greater than those for spontaneous mutation at either the aprt or hgprt loci in the same cell line (Table 2). Despite the high background frequencies of $FUdR^r$ colonies, significant dose-dependent increases in mutation frequency were observed with this genetic marker after treatment with known mutagens (Fig. 3). Furthermore, for most of the mutagens we have tested in the CHO-AT3-2 system, more mutations appear to be induced at the tk locus as a function of mutagen dose than at either the aprt or hgprt loci, or in the genes involved in ouabain resistance (16; Adair & Carver, manuscript in preparation). Clive et al. (26) reported that the tk locus was, on the

average, nearly 10 times more mutable than was the hgprt locus, for 13 agents tested in the L5178Y TK $^{+/-}$ mouse lymphoma system. Several carcinogens that were only weakly or non-mutagenic at the hgprt locus were found to be strongly mutagenic at the tk locus. Clive (26) has suggested that the L5178Y system, utilizing the tk locus, might be allowing the detection of TK-deficient mutants with gross chromosomal lesions as well as those generated by base-pair substitutions or small deletions. The extraordinarily high rate of spontaneous mutation at the tk locus in CHO-AT3-2 cells may reflect a position effect or "hot spot" for mutations or chromosomal events such as mitotic recombination, segregation, or non-disjunction. Further investigation of this system may provide insight into the involvement of extra-mutational events in the expression of genetic damage in somatic cells.

Cell density parameters. A major technical consideration in mutagen testing assays is the dependence of mutant selection upon wild type cell density, i.e., fewer mutants can survive in high density cultures because of metabolic cooperation (contact feeding). Cell density effects have been previously quantitated for several drug resistance markers in other cell lines (32,34,52,67,71,78,80,88,92). However, to establish optimal cell plating density conditions for the selection of $AA^r$, $AG^r$, $TG^r$, $OUA^R$, and $FUdR^r$ mutants in our cell line, the mean critical density $MCD_{50}$ (34), of CHO-IB-2 and CHO-AT3-2 cells for each drug resistant phenotype was determined.

Reconstruction experiments (Fig. 4), and determinations of spontaneous mutant frequencies as a function of cell plating density (Fig. 5), indicated that critical cell densities for TG, AG, and AA were $7.5 \times 10^3$ cells per $cm^2$, $3.0 \times 10^4$ cells per $cm^2$, and $1.3 \times 10^4$ cells per $cm^2$, respectively. Our cell density data for AA resistance are very similar to those reported in a detailed study of CHO-K1 by Dickerman and Tischfield (34) and do not show the density independence of $AA^r$ previously reported in another CHO cell line (52). Critical densities ranging from $4 \times 10^2$ to $4 \times 10^4$ cells per $cm^2$ for AG-resistance (32,41,51,71,78, 97), and from $6 \times 10^3$ to $1.4 \times 10^6$ cells per $cm^2$ has been reported for TG- resistance (27,31, Corsaro and Migeon as calculated in 34,58,71). The selection of $tk^{-/-}$ phenotypes was not dependent upon cell density over the range of 2 to $20 \times 10^3$ cells per $cm^2$. In general, cell lines plated in semi-solid agar substrates, e.g. L5178Y, S49, are not thought to show cell-to-cell cooperation in mutant selections (27,41). However, a recent report (67), indicated that BUdR (but not TFT) selection of L5178Y mutant cells in agar showed decreased recovery at $4 \times 10^4$ cells per $cm^2$.

Concentration of Selecting Drug. Cumulative data suggest that AG must be used at concentrations $\geq 30$ µg/ml to select reproducibly bona fide $hgprt^-$ mutants (12,39,41,53,78). Since selection of $TG^r$ mutants is affected by metabolic cooperation at densities approximately 4-5 fold lower than $AG^r$ (Figs. 4, 5a), we initially attempted to optimize the $AG^r$ selection and obtain quantitative, reproducible data. Although mutant selection can be improved by using 30 to 40 µg/ml AG, selection of $hgprt^-$ mutants with the CHO-IB-2 cell line is more definitive and quantitatively

reproducible when TG (2-4 μg/ml) is used as the selecting agent, an observation consistent with results reported in other cell lines (47,58,88,95). Furthermore, as shown in Fig. 12, the spectrum of residual HGPRT activity is much broader for mutants selected by resistance to AG; the resulting heterogeneity in colony size makes quantitation more difficult and requires extended incubation of mutant colonies (up to 18 d). Therefore, we prefer TG for selecting HGPRT-deficient mutants in our system. Azaadenine at 40 to 80 μg/ml effectively selects APRT-deficient mutants. While BUdR used at 150 μg/ml avoids most of the background haze associated with abortive phenotypes, the quantitation of TK-deficient mutants is more satisfactory with FUdR at 1 to 3 μg/ml; very definitive data are also obtained with TFT at 1 to 3 μg/ml.

Expression Time. Optimization of expression time is a major factor in the reproducible selection of drug-resistant mutants (48,84,89). Although expression time is generally expressed either as days or population doublings (or cell generations), both chronological time and cell division seem to contribute to expression (32,48,54). The HGPRT activity in a new presumptive mutant (and consequently its potential sensitivity to cytotoxic selecting agents) decreases as a function of both degradation of preexisting enzyme and dilution by cell division (48). Althogh HGPRT is apearently degraded even in contact-inhibited, nondividing cell populations (48), reports also indicate that maximal mutation frequencies are not achieved when cells are maintained in a nondividing state (54). Cox and Masson (32) presented evidence suggesting that cell doubline parameters may be more important than chronological time, at least in the expression of radiation-induced mutation at the hgprt locus. With direct, in situ expression of mutagenized cell populations, prolonged expression times are not practical because of cell-to-cell density effects and metabolic cooperation. With the replating method, the optimal expression time generally extends over at least several generations (1,13,32,48,69,74,96).

In CHO-AT3-2 cells, the optimal expression time depends on both the drug-resistant marker and the mutagen used (Figs. 8,9,10). Mutagen-specific variation in expression time has also been reported for diploid human lymphoblasts (91), in which MNNG-induced TG resistance was expressed by 6 generations but cells treated with ICR-191 required 12 to 16 generations before full expression had occurred. Shapiro and co-workers (82) reported maximal expression of $TG^r$ mutants after 2 to 6 generations, but expression times from 2 to 20 d have been reported by other investigators (1,27,32,48,58,74,82,89,91,96). In CHO-AT3-2, 6 to 8 d (8 to 10 generations) were required for maximal expression of $TG^r$ mutants (Fig. 9).

Optimal expression times for AG-resistance ranging from 3 to 7 d have been reported (32,39,51,69). Khalizev, et al. (54) reported that 2 to 3 doublings were sufficient for maximal expression of the $AG^r$ mutants phenotypes in Chinese hamster cells, but our data for CHO-IB-2 indicated that 5 to 7 doublings were required for optimal expression of $AG^r$ mutants induced by EMS or MNNG (data not shown).

In CHO-AT3-2 cells, 2 to 4 d are needed for full expression of $AA^r$ phenotypes (Fig. 8). For OUA resistance, maximal expression reportedly occurred in 1 to 4 d (27), while in our system 3 d (3 to 4 doublings) were sufficient for expression of most $OUA^R$ mutants (Fig. 10a). The mutant frequency was constant over the 10 d, whereas Buchwald (9) observed a decrease in the frequency of $OUA^R$ mutants after 2 generations of expression. For the tk locus, the optimal conditions for mutant expression are complex and mutagen-specific. For the mutagens studied, the maximal observed mutant frequency varies from 1 to 3 d (Figs. 10b, 10c). Previous reports indicated 2 to 3 d for mouse lymphoma (26), 3 d for V79 cells (80), and a constant 2 doublings (generations) for BHK cells treated with EMS (10).

Optimal expression time clearly varies for different mutagens, and the time for maximal expression observed for a few standard mutagens like EMS may be of limited value in predicting the optimal expression time for other classes of mutagens or for complex mixtures. If quantitative comparisons are to be made between mutagens, optimal expression time may need to be established for each mutagen, e.g., BP vs. DMN at the tk locus.

Dose Response Relationships at Different Genetic Markers. When the observed frequency of $AA^r$, $AG^r$, $TG^r$, and $OUA^R$ mutants per viable cell is compared for CHO-IB-2 as a function of molar dose (Table 6), both NQO and MNNG are more effective than EMS (approximately 200 to 350 fold and 100 to 500 fold, respectively). However, when the induced frequency of mutants per viable cell is compared for the various markers as a function of log cell survival, NQO and EMS are 2 to 7 and 3 to 9 fold more mutagenic than MNNG (Table 6). Thus, as previously suggested (29,77), when analyzed on the basis of cell survival, MNNG is not a particularly effective mutagen in mammalian cells.

As a function of applied molar dose, the mutational response at the hgprt locus in CHO-AT3-2 cells had a slope of $5.2 \times 10^{-4}$ mutants per viable cell per mM EMS, compared to a slope of $4.3 \times 10^{-4}$ previously reported for this locus by Hsie and co-workers (45) at similar 16 h treatment times. Slopes for $AA^r$, $OUA^R$, and $FUdR^r$ mutants after 8 d expression were $4.1 \times 10^{-4}$, $1.3 \times 10^{-4}$, and $1.3 \times 10^{-3}$, respectively (3).

When the mutational response for EMS is plotted as a function of cell survival, the observed frequency of $AA^r$, $TG^r$, and $OUA^R$, mutants by EMS decreases at survivals  0.50 (Fig. 11), as was the case in previous experiments using CHO-IB-2 cells (Table 6). However, induction of $FUdR^r$ mutants by EMS increased linearly as a function of the logarithym of decreasing survival, as did induction of $AA^r$ or $TG^r$ mutants by DMN.

Specificity and sensitivity of the multiple markers. Significant increases in mutation frequency were observed for all four drug resistance markers following treatment of CHO-AT3-2 cells with EMS, DMN, or BP (Fig. 11), but locus-specific differences in sensitivity to these mutagens were noted. Under optimal expression conditions, the tk locus consistently showed the greatest mutational response as a function of cell survival with

induction by DMN = BP ≥ EMS. The tk locus was much more sensitive than the other loci to mutation induction by DMN and BP, but only slightly more sensitive to mutation induction by EMS. With DMN and BP, the slopes for mutation induction for $FUdR^r$ (Fig. 11) were about 25 to 40 fold greater than those for $AA^r$ or $TG^r$, and over 100 fold greater than those for $OUA^R$. With EMS, mutation induction slopes for $FUdR^r$ were still about 3 to 4 fold higher than those for $AA^r$ or $TG^r$, and about 12 fold higher than those for $OUA^R$. EMS, however, was much (7 to 9 fold) more effective in inducing $AA^r$, $TG^r$, and $OUA^R$ mutants than either DMN or BP.

Of the four genetic markers employed in CHO-AT3-2 mutagenesis assay system, the tk locus has consistently shown the greatest mutational response to positive mutagens (Ref. 3, Fig. 11). The magnitude of induced mutation at this locus suggest that FUdR selection in the CHO-AT3-2 system may detect a broader range of genetic lesions than the other drug-resistance markers. In the L5178Y $TK^{+/-}$ mouse lymphoma system, the tk locus appears to be approximately ten times more mutable than the hgprt locus (26), and Clive and co-workers have postulated that their system may be detecting TK-deficient variants arising from gross chromosomal lesions as well as those generated by base-pair substitution, frame shift, or small deletions. In our CHO-AT3-2 cells, as in the L5178Y $TK^{+/-}$ system, phenotypic expression of mutants resistant to thymidine analogs was rapid, with maximal frequencies generally attained within 1 to 3 d after mutagen treatment (Fig. 10). Mutant frequencies typically decline with longer expresstion times. In both systems, cell isolates resistant to thymidine analogs have little or no detectable TK activity (22,25) as would be expected in the case of deletions or gross chromosomal lesions.

The rapid expression kinetics for induced $FUdR^r$ mutants may facilitate the detection of mutants with gross chromosomal anomalies or large scale DNA damage affecting cell growth rate. Such gross genetic lesions might not be detected using either the $OUA^R$ marker (a functional $Na^+$-$K^+$-ATPase is required for cell survival) or the $TG^r$ marker (expression of $TG^r$ phenotypes requires long expression times). Moreover, the gene for tk may be unusually susceptible to mutation. The high frequencies of $FUdR^r$ observed in unmutagenized CHO-AT3-2 cell populations are the result of an exceptionally high, apparent rate of spontaneous mutation at the tk locus (3). The apparent susceptibility to mutation of the tk locus in CHO-AT3-2 cells may reflect chromosomal events such as mitotic recombination, segregation, or nondisjunction. Position effects in lower eucaryotes and "hot spots" in microbial systems are not uncommon. Since the $FUdR^r$ marker may allow detection of a broader range of genetic lesions than the other drug resistance markers, use of the tk locus in the CHO-AT3-2 mutagenesis assay enhances the potential sensitivity of the system.

Validation of mutant phenotypes. As shown in Fig. 12, the spectrum of residual enzyme activities of $AG^r$ mutants is more heterogeneous than that for $TG^r$ mutants. Although many of the mutants selected with AG display stable characteristics, the observed heterogeneity probably contributes to technical problems with the selection of $AG^r$ phenotypes. There was no

statistical difference in the residual enzyme activities in EMS-, MNNG-, and NQO-induced mutants (data not shown). Mutants selected with FUdR, BUdR, and TFT appear to have identical enzymatic properties, displaying ≤ 1% WT TK activity, sensitivity to HAT, and cross resistance to all three selecting drugs. The results in Fig. 12 indicate that a very high percentage of the spontaneous and induced drug-resistant clones scored as mutants have substantially diminished enzyme activity at the selected locus.

The Multiple Marker Mutagenesis Assay System. Systematic determination of optimal conditions for the phenotypic expression and selection of $AA^r$, $FUdR^r$, $OUA^R$ and $TG^r$ mutants (15,16; Adair & Carver, manuscript in preparation), and the development and application of a multiple-marker mutagenesis assay system employing CHO-AT3-2 will facilitate quantitation and direct comparison of the extent of mutational damage at these four defined genetic loci. The CHO-AT3-2 cells combine favorable growth characteristics (e.g., short doubling time, excellent cloning efficiency, and the ability to grow in either monolayer or suspension culture) with a near diploid genome that facilitates genetic and cytogenetic analysis. As an *in vitro* mutagenesis assay syste, the CHO-AT3-2 cell line offers the unique capability of measuring mutation induction at the autosomal *aprt* and *tk* loci as well as in the genes involved in ouabain resistance or at the X-linked *hgprt* locus. The assay thus combines the attributes of the two major *in vitro* mammalian-cell mutagenesis systems currently being used Furthermore, it offers the only CHO assay presently using the *tk* locus and facilitates comparison with results from the L5178Y mouse lymphoma system (20,22,24,25,26). Such comparisons will be invaluable in determining species variation among short-term, *in vitro* mutagenesis assays. To develop this system and confirm its potential, we have: (a) selected CHO stock lines heterozygous at the *aprt* and the *tk* loci (necessary for single-step selection of autosomal recessive mutations), (b) tested stock cell lines for optimal plating density, expression time, and concentration of selecting drugs, (c) validated the assay with data for induced mutant frequencies after treatment with the direct mutagens EMS, MNNG, and NQO and promutagens DMN and BP, (d) quantified and defined the dose response, and (e) confirmed the mutational events by assays for enzyme activities in mutant clones isolated from the above experiments.

Because the mechanisms by which mutational damage is incurred may vary considerably for different agents, and genetic loci may differ in their sensitivity to those agents, systems employing multiple markers may be more effective in detecting potentially mutagenic agents. The operating parameters for our multiple marker mutagenesis assay are now well-defined and the system can be applied to testing other known mutagen classes as well as to screening samples containing mixtures of potentially mutagenic agents. Coupled with the metabolic activation methods, the system can be used to screen crude mixtures and effluents of environmental concern.

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## GLOSSARY

| | |
|---|---|
| $AA^r$ | cell phenotype, resistance to AA |
| $AG^r$ | cell phenotype, resistance to AG |
| $aprt^{+/+}$ | cell genotype, with two genes for functional APRT enzyme |
| $aprt^{+/-}$ | cells with only one gene (heterzygous) for functional APRT enzyme |
| $aprt^{-/-}$ | cells with no gene for functional APRT enzyme |
| CHO-IB-2 | cell line heterzygous at the aprt locus |
| CHO-AT3-2 | cell line heterzygous at the aprt and tk loci |
| $hgprt^+$ | cell genotype, with a gene (X-linked) for functional HGPRT enzyme |
| $hgprt^-$ | cells without a gene for functional HGPRT enzyme |
| $OUA^R$ | cell phenotype, resistance to OUA |
| $FUdR^r$ | cell phenotype, resistance to FUdR |
| PE | ratio of viable clls to cells plated; mean plating efficiency |
| $\bar{S}$, cells | surviving cell fraction; mean cell survival relative to controls |
| $TG^r$ | cell phenotype, resistance to TG |
| $tk^{+/+}$ | cell genotype, with two genes for functional TK enzyme |
| $tk^{+/-}$ | cell genotype, with only one gene for functional TK enzyme |
| $tk^{-/-}$ | cells with no gene for functional TK enzyme |
| WT | wild type cells ($aprt^{+/+}$, $hgprt^+$, $tk^{+/+}$, ATPase with normal sensitivity to ouabain) |

## TECHNICAL REPORT DATA

*(Please read Instructions on the reverse before completing)*

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This project is part of the EPA-planned and coordinated Federal Interagency Energy/Environment R&D Program.

16. ABSTRACT

Chinese hamster ovary (CHO) cell lines heterozygous at the adenine phosphoribosyltransferase (aprt) and thymidine kinase (tk) loci were isolated and used for single-step selection of spontaneous and induced mutants resistant to 8-azaadenine ($AA^r$), 6-thioguanine ($TG^r$), 5-fluorodeoxyuridine ($FUdR^r$), or ouabain ($OUA^R$). Mutation data are reported for direct mutagens ethyl methanesulfonate (EMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), 4-nitroquinoline-1-oxide (NQO) and for promutagens dimethylnitrosamine (DMN) and benzo(a)pyrene (BaP) activated by rat liver homogenates. Critical plating densities were established for $AA^r$, $TG^r$, and $FUdR^r$. Optimal expression of mutant phenotypes after mutation induction with EMS, DMN, or BaP were 2 to 4 d for $AA^r$, 6 to 8 d for $TG^r$, 3 d for $OUA^R$, and 1 to 3 d for $FUdR^r$. The induced mutant frequencies as a function of relative cell survival after treatment with EMS, DMN, or BaP showed locus-specific differences in sensitivity. Of 61 clonal isolates resistant to AA and assayed for APRT activity, 87% had $\leq 5\%$ wild type activity; of 30 $TG^r$ clones assayed, 83% had $\leq 5\%$ wild type HGPRT activity. Of 42 $FUdR^r$ clones assayed, 41 had $< 1\%$ wild type TK activity. Fifty clones selected in medium containing FUdR displayed cross-resistance to 5-bromodeoxyuridine (BUdR) and trifluorothymidine (TFT) and all were sensitive to HAT (amethopterin-hypoxanthine-thymidine) medium. The tk locus showed the largest mutational response as a function of relative cell survival after mutagen treatment.

17. KEY WORDS AND DOCUMENT ANALYSIS

| a. DESCRIPTORS | b. IDENTIFIERS/OPEN ENDED TERMS | c. COSATI Field/Group |
|---|---|---|
| induced mutants<br>mutation rate | | |

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